Biological and Behavioural Markers of Smoking Reduction

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The research associated with this thesis abides by the international and Australian codes on human and animal experimentation. The research was reviewed and approved by the Tasmania Health and Medical Human Research Ethics Committee (approval reference number: H0013619, H0011574).

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Abstract

Tobacco harm reduction involves strategies designed to reduce the harms associated with tobacco use, for example by cutting down cigarette consumption and thereby reducing exposure to the risks associated with smoking. In addition to potentially reducing the harms of continued smoking, there is evidence that some harm reduction strategies are positively associated with quitting smoking entirely. A variety of interventions exist that can be used to aid both cessation and smoking reduction. However, there are limitations with using current treatments, with most attempts to change smoking behaviour ultimately ending with relapse. Numerous limitations of current treatments have been noted in the past, including that existing nicotine replacement products may not provide sufficient nicotine, and / or that the time course of nicotine delivery may not be optimal for promoting reduction. The broad objective of this thesis is to understand how smoking reduction influences smoking behaviours and cessation outcomes as well as smoking-related harm exposure.

To achieve this aim, firstly, knowing the content of nicotine in supplementary products can help with interpreting the outcomes of clinical trials and with improving product design. Vaporised nicotine products (VNPs) – commonly known as electronic cigarettes – are increasingly being used by smokers to reduce their smoking, and there is some evidence that they can be effective cessation aids. A key issue with testing the effectiveness of these products, however, as been quantifying the variability in the amount of nicotine contained in products. As such, an assay was developed and applied to determine the nicotine content in one brand of VNPs which consisted of a fibrous pad in the cartridge. In addition to finding substantial variation between product batches, we also found that the measured nicotine content in the cartridge was lower than the stated content on the product label. The accurate

determination of the quantity of nicotine in VNPs has important implications for both consumer safety and the further study of these devices.

Secondly, smoking reduction is not only a reduction in the number of smoked cigarettes, but also an expected reduction in exposure of smoking-related harm. Biological markers can be used to indicate the intake of nicotine and smoking-related harm. 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) is a metabolite of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), which is a tobacco specific carcinogen. As such, NNAL is considered a biological marker associated with tobacco-related harm. To measure the levels of NNAL in smokers' urine, solid phase extraction (SPE) assay has been used in previously reported studies. However, the sample preparation procedures required with these SPE assays are complicated and time consuming, limiting their use. A simple and efficient liquid-liquid extraction assay combined with ultra-performance liquid chromatography with tandem mass spectrometry (UPLC-MS/MS) was designed in this study, and applied to the smokers' urine to measure changes in harm exposure over the course of a quit attempt. As expected, the NNAL levels were significantly decreased following the quit attempt. This supports the use of NNAL as a biomarker of tobacco-related harm exposure and harm minimisation. This assay is an improvement over existing SPE-based methods in that it is substantially less complicated while maintaining adequate sensitivity and is a straightforward approach to measuring NNAL levels. The assay also demonstrated that using enzymatic hydrolysis approach of NNAL-glucuronide – had taken by other studies – as a technique for measuring the aglycone was clearly unreliable. There was a potential for significant loss of either free NNAL and NNAL aglycone. NNAL-glucuronide standards are recommended to utilise for proper validation of indirect methods such as enzymatic hydrolysis approach to measure total NNAL (free + glucuronide).

Finally, we explored the mechanism through which two medications – specifically varenicline and nicotine patch – promote reduction. There has been growing research interest in using nicotine replacement medications to aid smoking reduction prior to a quit attempt. Gaining a better understanding of how treatments influence smoking reduction may allow for better tailoring of treatments and, ultimately, better cessation outcomes. The objective of the study was to test the effects of the pre-quit use of varenicline and nicotine patch on smoking rate and satisfaction with smoking. Participants in the two pre-quit treatment groups reported significant reductions in both their satisfaction with smoking and smoking rate from baseline to the end of pre-quit period; participants in the standard patch group, which started to use of nicotine patch at a quit attempt day, did not. The observed reduction of smoking rate was associated with the satisfaction with smoking, although the mediation effect of satisfaction was weak. As such, monitoring reductions in satisfaction does not appear to be a viable method of evaluating responsiveness to treatment and, or for tailoring.

In summary, this research developed a sensitive and efficient assay to determine nicotine content in supplementary nicotine products, providing valuable data for further research on these products. Furthermore, an assay for determination of NNAL levels in smokers' urine was developed, which was applied to determine the reduction of smoking-related harm exposure in a smoking cessation study. Finally, this research demonstrated that pre-quit treatment use led to reductions in satisfaction with smoking and the smoking rate, and the satisfaction was associated with the reduction in smoking rate. Monitoring such reductions may prove to be useful in evaluating responsiveness to treatment, risk of harm, and allow for tailoring of treatment.

List of Abbreviations

CO	carbon monoxide
COPD	chronic obstructive pulmonary disease
CPD	cigarettes smoked per day
DCM	dichloromethane
DLLME	dispersive liquid-liquid micro-extraction
DLLME-SFO	dispersive liquid-liquid micro-extraction based on the solidification of floating organic droplet
EMA	ecological momentary assessment
ENDS	electronic nicotine delivery system
FDA	Food and Drug Administration
GC	gas chromatography
GC-TSD	gas chromatography with the thermionic specific detector
HCl	hydrogen chloride
HPLC	high performance liquid chromatography
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LoD	limit of detection
LLoQ	lower limit of quantification
MIP	molecularly imprinted polymer
MRM	multiple reaction monitoring
nAChRs	neuronal nicotine acetylcholine receptors
NaOH	sodium hydroxide
NAB	N'-nitrosoanabasine
NAT	N'-nitrosoanatabine
NNN	N'-nitrosonornicotine
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNAL	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol
NNAL-d_3	deuterated NNAL
NRT	nicotine replacement therapy
PQP	pre-quit patch
RSD	relative standard deviation
SD	standard deviation
SPE	solid phase extraction
TSNAs	tobacco-specific nitrosamines
UPLC	ultra-performance liquid chromatography
UPLC-MS/MS	ultra-performance liquid chromatography with tandem mass spectrometry
VNPs	vaporised nicotine products
WHO	World Health Organization

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Chapter 1: Introduction

Chapter 1

Cigarette smoking remains one of the greatest causes of preventable death and disease in the developed world [1]. The most obvious solution to this problem is complete cessation from tobacco product use. However, despite years of attention from the public health community, rates of smoking remain unacceptably high; this is not only true in the developing world [2], but also in developed countries, where smoking rates remain high, especially among vulnerable pockets of the community (e.g., those with mental health issues [3, 4]).

The recalcitrant nature of smoking has led many in the tobacco control community to entertain the virtues of harm reduction strategies. Harm reduction can be defined as a policy, strategy or specific intervention to minimise the adverse consequences associated with the continuing drug use [5]. Tobacco harm reduction typically involves cutting down – but not necessarily completely stopping – the consumption of traditional combustible cigarettes, thereby reducing exposure to the risks associated with smoking. Reduction can theoretically be accomplished through restraint strategies (e.g., willpower) alone, but sustained reduction has proved difficult to maintain, presumably because of dependence on nicotine. An alternate strategy is to replace, or substitute, the nicotine forgone through reduction via another source, for example using nicotine replacement products such as nicotine gum or nicotine lozenges [6, 7]. More recently, researchers have explored the potential impact of alternate additional nicotine delivery systems, most notably vaporised nicotine products (VNPs), colloquially referred to as electronic-cigarettes (or ‘e-cigarettes’). Regardless of the method of reduction used, however, the goal remains the same: to reduce the consumption of traditional combustible cigarettes, thereby reducing tobacco-related harm.

While it seems self-evident that smoking fewer cigarettes would be better than smoking more, quantifying the risks and benefits of tobacco harm reduction, either at a

socially or an individual level, is complicated. Firstly, there is no known safe level of smoking: every cigarette smoked increases an individual's risk of tobacco-related disease [8, 9]. Furthermore, even assessing reduction itself is complicated as the exact harm associated with each cigarette is not consistent: smokers can compensate for reduction by smoking each remaining cigarette more intensely; thus, simply counting the number of cigarettes per day (CPD) that an individual consumes is a crude measure reduction, let alone harm reduction.

To better quantify reduction, tobacco researchers have turned to biological markers of tobacco exposure. Biological markers can be used to measure nicotine and its metabolites in biological fluids, thereby assessing exposure to tobacco products. Similarly, researchers have developed methods for assessing the constituents of products themselves [10] as a method of measuring intake and exposure. A variety of assays currently exist for the measurement of both biological markers of tobacco exposure and the constituents of tobacco products. These assays have been used by researchers to access smoking and nicotine intake. However, no current measure is a perfect proxy for actual exposure and existing measures have known limitations. The objective of this thesis was to develop improved measures of exposure – both in terms of measuring biological markers of actual exposure, and in terms of the constituents of products themselves – and to use these measures to studying smoking behaviour and its consequences.

The whole thesis is divided into eight chapters. Chapter 2 of the thesis is a literature review covering key topics including: smoking prevalence and smoking related harm; tobacco harm reduction; and current methods of accomplishing reduction used in harm reduction and their limitations.

Chapter 1

With the high interest in tobacco harm reduction, a variety of medical nicotine products are utilised to assist harm reduction. However, if the nicotine content in the products is unknown or inconsistent, it is difficult to confidently say that the products can be useful in assessing smoking reduction. This is particularly important given that VNPs are unregulated and produced with unknown manufacturing standards [11]. With this concern in mind, Chapter 3 of this thesis includes the development of an assay to determine the nicotine content in VNP products, and the application of this assay on VNP products to investigate whether the nicotine content is consistent with the label and consistent between product batches (Study 1). Knowing the nicotine content would allow better interpretation of clinical outcomes when using these products in smoking reduction studies.

To assess the success of the harm reduction, simply measuring the number of cigarettes smoked is not sufficient. Smokers can titrate the amount of nicotine that they extract from individual cigarettes; thus, they can compensate for skipped cigarettes by smoking the remaining cigarettes more intensely, potentially obviating any possible benefits of reduced intake. To overcome this issue, biological markers can be used to accurately assess exposure and nicotine intake. Nicotine is extensively metabolised by a variety of liver enzymes. Cytochrome P450 2A6 is one of the main nicotine metabolising enzyme in humans. Nicotine and its metabolites are further metabolised through glucuronidation process, which is facilitated by uridine 5'-diphospho-glucuronosyltransferase. Nicotine also forms other chemicals during curing process. Tobacco-specific nitrosamines (TSNAs) are one group of carcinogens in tobacco products, and are also presented in tobacco smoke. 4-(Methylnitrosamino)-1-(3-pyridyl)-1-1butanone (NNK), N'-nitrosonornicotine (NNN), N'-nitrosoanabasine (NAB), and N'-nitrosoanatabine (NAT) are formed by oxidation and nitrosation of nicotine. 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) is the major

metabolite of NNK, which is formed from nicotine and related compounds by a nitrosation reaction that occurs during the curing of tobacco. Total NNAL (free + glucuronide) is not presented in cigarette smoke, therefore it is considered an effective biomarker of NNK uptake and hence tobacco exposure. The long biologic half-life of NNAL also can be used to determine the exposure of tobacco-related harm. As detailed in Chapter 4, current methods for measuring NNAL levels involve complex sample preparation procedures. An easy but effective assay is required to measure NNAL levels in smokers' urine. Chapter 4 of this thesis contains the development of an assay for determining NNAL levels in human urine (Study 2), and Chapter 5 of this thesis contains the application of this NNAL assay to smokers' urine that had been collected from a smoking cessation study (Study 3).

Smoking reduction, as well as being seen as a potentially beneficial end in and of itself, research also suggests that it may promote complete cessation. In the final data chapter (Chapter 6) of the thesis I attempt to gain a better understanding of the impact of reduction using data drawn from a clinical trial involving two treatments, nicotine patches and varenicline (Study 4). The purpose of the trial was to examine the effects of pre-quit, or pre-cessation, treatment on daily smoking rate and satisfaction with smoking, and test the relationship between these variables during the two weeks leading up to a quit attempt. Ecological momentary assessment (EMA) was used to monitor the smoking behaviour, affect and activities in real-time.

The final two chapters of this thesis focus on a general discussion and conclusion of the developed assays for determining smoking harm reduction: the determination of nicotine in substitute products, and biological markers of actual smoking harm exposure. These measures assist the study of smoking behaviour and its consequences.

Chapter 2: Literature Review

Chapter 2

2.1 Background

Each year, approximately five million people worldwide die from direct tobacco use and 600,000 non-smokers die from exposure to second-hand smoke [1]. Tobacco smoking is a leading risk factor for tobacco-related chronic diseases, including respiratory disease, cardiovascular disease and different types of cancers, especially lung cancer [9]. More than 80% of the chronic lung diseases, including respiratory infections, poor lung compliance, chronic obstructive pulmonary disease (COPD) and worsening of asthma, are attributed to smoking [12]. Indeed, tobacco smoking is the most important risk factor for COPD [13]. Tobacco-related COPD is the fourth most common cause of chronic disability and death in developed countries. Exposure to second-hand smoke is also a risk factor for respiratory diseases in children and adults who do not smoke. Long-term exposure to second-hand smoke can increase lung cancer risk by up to 30% and coronary heart disease in non-smoking adults [14, 15].

Quitting smoking is the most effective intervention for current smokers to reduce morbidity and mortality of smoking-related diseases. For instance, studies found that smoking cessation reduced 36% of mortality risk in patient with coronary heart disease [16] and significantly improved an individual's prognosis after a cardiac event [17]. With COPD, smoking cessation is considered as an effective treatment for reducing the progression of this disease [18, 19]. A long-term follow-up study also indicated that quitting smoking was associated with a slower lung function decline compared with continuing smokers [20]. Smoking cessation also reduces COPD mortality risk ranging from 32-84% compared with continuing smokers [21]. The majority of smokers would like to stop smoking, thereby reducing their personal risk of developing smoking-related diseases [8, 9]. More than half of US smokers have tried to quit smoking. Similarly, in Australia, approximately 75% of

smokers have tried to change smoking behaviour in the last 12 months [22]. Previous studies have also indicated that about 40% of smokers made quit attempts in a given year [23], and the quit attempt lasted for a short or long-term period of time [24, 25]. Even though many smokers make quit attempts every year, most of these quit attempts ultimately fail. To reduce smoking rates and reduce smoking-related harm, researchers and policy makers have increased their interest in exploring tobacco harm reduction strategies.

2.2 Tobacco harm reduction

Nicotine is considered to be the primary addictive component driving the consumption of tobacco, yet the majority of smoking-related harm themselves are not caused by nicotine itself [26]. When burning tobacco, the smoke contains an extraordinarily complicated mixture, which includes over 60 known carcinogens. Tobacco smoke exposure is the major risk of causing cancer, heart disease, respiratory disease and other deadly consequences [27]. Considering the tobacco-related morbidity and mortality, tobacco control and promotion of public health are an important part of the global health strategy [28]. Harm reduction can be defined as a policy, strategy or specific intervention to minimise the adverse consequences associated with continuing drug use [5]. Tobacco harm reduction involves cutting down the consumption of traditional combustible cigarette but not necessarily completely stopping nicotine use, thereby reducing exposure to the risks associated with smoking [27]. As well as *potentially* reducing the harms associated with smoking, reducing the number of cigarettes smoked per day may also prompt quitting itself [29, 30]. In other words, harm reduction approaches may result in increased motivation to quit completely [31-35].

2.2.1 Tobacco control strategies

In Australia, governments work in collaboration with non-government agencies to conduct tobacco control strategies designed to reduce tobacco use [36]; the same is true in other countries [1]. One of the effective tobacco control strategies is increasing tobacco product taxation. Studies have shown about 44% of smokers changed their smoking behaviour due to the high cost of tobacco products [37]. Increased taxes promote an increased proportion of smokers attempting to quit, and decrease the number of smoked cigarettes [38, 39]. Another effective tobacco control strategy is a smoke-free environment policy. This policy protects individuals from exposure of second-hand smoke [40] and prevents uptake of smoking among youth [41]. In the last two decades, a new tobacco control strategy has been established by applying anti-smoking and health warning message on cigarette packages to discourage the uptake of cigarettes and to encourage quitting; the effects of these warning messages on smokers are still under investigation [42]. These tobacco control strategies aim to help with reducing the prevalence of smoking and its associated health, social and economic costs, which have been in place for a number of decades [43]. In general, with these tobacco control strategies, daily tobacco smoking among the general Australian population has declined to historically low levels [43]. Data from 2013 show that, among people aged 14 or older, daily smoking declined from 15.1% in 2010 to 12.8%; similarly among adults (aged 18 or older) daily smoking declined from 15.9% in 2010 to 13.3% [43]. Tobacco control strategies have made a significant impact on cigarette consumption and smoking rates, predominately by preventing non-smokers from starting to smoke. But, as will be described below, sustained reduction among current smokers has proved difficult to maintain, predominantly because of dependence to nicotine.

2.2.2 Smoking cessation treatments

Nicotine is an addictive substance binding to neuronal nicotine acetylcholine receptors (nAChRs) thus causing nicotine dependence, which is the primary determinant of persistent tobacco smoking [44]. Nicotine dependence is psychological and / or physical. Stopping nicotine intake – either partially or completely – can cause nicotine craving, anxiety, irritability, confusion, and difficulty sleeping, which are categorised as nicotine withdrawal symptoms [45]. Regular nicotine intake is required to maintain a nicotine level above a threshold point (the minimum amount of nicotine that produces reward effect). However, as there is no known safe level of smoking [8, 9, 46], complete quitting is clearly the most effective method of reducing smoking-related diseases. While most smokers report wanting to quit, sustained abstinence is elusive for most smokers. For example, in 2010, approximately 29% of Australian smokers reported they had tried to quit smoking but were unsuccessful, and 40% of smokers reported they had reduced their smoking [37].

Quitting smoking is a huge challenge for smokers because of the addictive property of nicotine. In order to sustain a quit action and achieve cessation, another approach is to replace – or substitute – the nicotine via another source, for example using pharmacotherapy nicotine products, such as nicotine replacement therapy (NRT), or aid with non-nicotine medical therapy, such as varenicline and bupropion. Non-pharmacological smoking cessation services are also an effective way to support smoking cessation.

2.2.2.1 Nicotine Replacement Therapy (NRT)

Nicotine replacement products are effective interventions for smoking cessation [47]. NRT is developed as a method of replacing the nicotine that a smoker would otherwise obtain from smoking. It is believed that replacing the nicotine forgone during abstinence would reduce

withdrawal symptom severity and thereby make abstinence easier to sustain (see [48]). A variety of NRT products are available today, including nicotine patches, lozenge, gum, inhalator, and nasal spray.

Nicotine gum is the earliest available type of NRT [49]. Nicotine is absorbed through the buccal mucosa, resulting in blood concentration approximately half that produced by smoking a cigarette [50]. However, there are limitations of using nicotine gum, such as oral and gastric side effects [51]. Other types of NRT subsequently were developed. Nicotine patch is a widely used form of NRT [52, 53]. The patch consistently and steadily delivers nicotine through the dermis into the blood stream. With nicotine patch treatment, the cessation rate can be increased by approximately two times compared with placebo treatment [54]. Other types of NRT, such as nicotine nasal spray, nicotine lozenge, have also been successfully developed for smoking cessation [49, 54].

2.2.2.2 Varenicline and Bupropion – non-nicotine medical therapies

Varenicline, a selective nicotine receptor partial agonist, is the newest medical smoking cessation product and more effective than using a single type of NRT [55]. It inhibits dopaminergic activation produced by smoking while simultaneously providing relief from the craving of smoking [56]. Varenicline treatment is started prior to the target quit day, and there is evidence that this promotes gradual reduction of smoking prior to quitting [57].

Bupropion is another commonly used non-nicotine therapy to aid smoking cessation, initially developed as an antidepressant [58]. It is a selective re-uptake inhibitor of dopamine and noradrenalin [59], and also acts as an antagonist of nicotine acetylcholine receptors [60], which reduces the craving to smoking and reduces symptoms from nicotine withdrawal [61].

Reviewed literatures indicated that bupropion increased the likelihood of a successful quit attempt after at least six months [62]. The effectiveness of bupropion is comparable to NRT [62], but less effective than varenicline [63, 64]

2.2.2.3 Non-pharmacological Smoking Cessation Treatments

In additional to quit smoking medications discussed above, a variety of non-pharmacological smoking cessation services are also available for smokers wishing to quit, such as educational materials provided at clinics, hospitals, and telephone support quit lines [36, 65]. These services – broadly referred to as behavioural support – typical comprise education and advice designed to aid smokers as they attempt to quit. Behavioural support interventions can be delivered in a range formats, from static, written materials through to tailored content provided via mobile phone applications. Combining behavioural support and pharmacotherapy can improve the chance of maintaining long-term abstinence [66].

2.2.3 Smoking reduction

As discussed above, smokers interested in quitting can chose from a variety of medications and services proven to improve their odds of achieving abstinence. Sadly, however, the absolute effectiveness of these tools is modest, with approximately 70% of aided cessation efforts ultimately ending in failure [55, 67]. One potential reason of the unsuccessfully quitting is the failure to use smoking cessation methods efficiently [52]; other factors also can influence the success of cessation. Nevertheless, it is clear that many smokers find it difficult to maintain complete abstinence, at least in the long term. For this reason, the tobacco control community has started to explore the benefits of smoking reduction.

Chapter 2

Smoking reduction can be considered one form of harm reduction in those are not currently ready to quit [34, 68]. Smoking reduction is self-explanatory: it involves encouraging smokers who are either unwilling or unable to quit to reduce the CPD over a period of time, either as an end it itself, or as an initial step toward eventually quitting completely [33, 69]. Research study surveys have found that many smokers were interested in gradually reducing their smoking prior to making a quit attempt [34, 68], and a Cochrane review of the literature concluded that gradual reduction can be an effective method of achieving abstinence [33]. Given this evidence gradual reduction is increasingly seen as a viable approach for achieving abstinence [70].

As with cessation, a variety of interventions have been evaluated as reduction aids. A Cochrane review of the literature concluded that NRT effectively aided smokers to reduce smoking compared to placebo [71]. For instance, nicotine gum has been used in a smoking reduction study. This study indicated that there was two-fold sustained smoking reduction rate with nicotine gum use versus with placebo [72]. Other types of NRT, including nicotine inhalator (an oral device by delivering nicotine through buccal mucosa [47, 73]) and nicotine lozenge [7], have been reported to be effective in gradual smoking reduction. Nicotine patches have also been used 1-2 weeks prior to a target quit day (known as "preloading" [74]), which assist smokers to gradually reduce smoking.

Interesting, data suggest that successfully reducing smoking aided with NRT is more likely to increase a quit attempt before the quit process [34, 47]. In other words, giving NRT prior to the quit attempt day may increase the likelihood of a successful quit attempt. Exploring the mechanism though which pre-treatment with nicotine patches influence smoking reduction is the focus of Chapter 6 of this thesis.

Finally, purely behaviour treatments have also been used to promote reduction [75]. However, the results of such treatments have been disappointing, with the majority of smokers unable to successfully maintain reduction in the longer-term.

2.2.4 Limitations of existing NRT reduction treatments

As noted above, numerous treatments exist that have been shown to aid reduction. There is substantial scope to improve the current offerings, however. Two key factors in the effectiveness of a nicotine replacement medication are the speed of nicotine delivery and the dose of nicotine obtained from NRT. Nicotine is absorbed into the bloodstream and delivered to the brain within 20 seconds after one inhalation of a cigarette [76], and the blood nicotine typically concentration ranges from 15-30 ng/L within 8 min [77, 78]. However, NRT products deliver nicotine substantially slower than smoking, with blood nicotine levels reaching a peak within 20 min [54, 79]. Even the fastest delivery product – nicotine nasal spray – still takes 10-15 min to reach the peak [80]. In addition, the amount of nicotine is not effectively absorbed. For instance, the nicotine intake level was lower while chewing gum compared to ad libitum, and this could be because nicotine was swallowed and underwent the first-pass metabolism [81]; and more frequent admission of nicotine gum is required [82]. In addition, even if the treatment could mimic the nicotine delivery that we see with smoking, it still would not replace cigarettes perfectly.

2.2.5 New nicotine delivery products

A new form of electronic nicotine delivery systems (ENDS), also known as vaporised nicotine products (VNPs), or colloquially referred to as electronic-cigarettes (“e-cigarettes”), was first introduced into cigarettes markets in 2004 [83]. VNPs are battery-operated devices capable of vaporising a nicotine solution instead of combusting tobacco and delivering

nicotine in an aerosolised form. The awareness and use of VNPs have grown rapidly over the past few years [84, 85]. Currently in the UK, there are over two million smokers using VNPs [86]. VNPs have become the most popular smoking products among youth in the US [87]. With the rapid increase in popularity, the design of VNPs has also rapidly updated. In the beginning, VNPs aimed to make people feel like they were having a normal cigarette [83]. Therefore, the VNPs were designed to resemble cigarettes and vaporise a liquid to generate a warm vapour or mist with each puff. The second generation of VNPs featured a larger capacity of batteries and the appearance looked like pens. Shortly after, VNP models were updated to third generation (also called 'mods', from modifications) [88]. The design and function have been modified to be more practical to meet the needs of VNP users. To date, the fourth generation of VNPs have emerged in the market. They feature automatic temperature control and adjustable dual airflow slots, which are more advanced and can deliver more nicotine (than the older three generation models).

There are many reasons behind the rapidly increasing prevalence of VNPs. One reason is that they are considered safer than tobacco cigarettes. The daily exposure to nitrosamines (carcinogens formed from tobacco cigarettes, also known as tobacco-specific nitrosamines), based on an average consumption of 15 cigarettes per day, is estimated to be higher compared to that found after use of VNPs [89]. Two tobacco-specific nitrosamines, 4-(methylnitrsamino)-1-(3-pyridyl)-1 butanone (NNK) and N'-nitrosonornicotine (NNN), are considered as major carcinogens that cause lung cancer. Other toxic chemicals such as heavy metals, carcinogenic polycyclic aromatic hydrocarbons and phenols have not been detected, with the exception of trivial amounts of mercury (0.17 ng per VNP) and traces of formaldehyde and acetaldehyde [90]. Research also evaluated emissions based on a toxicant emissions score and reported a score of 0 in VNPs compared with a score of 100–134 for

tobacco cigarettes. A UK panel of health experts concluded that, based on the available evidence, VNPs are at least 95% less harmful than cigarettes [91, 92]. However, the field is split in terms of their views on the safety of VNPs – and even their safety relative to traditional combustible cigarettes – with many study concluding that these products are not a safer alternative to smoking [93]. Work in this area is ongoing and will likely be contentious for years to come.

Apart from the potentially lower risk associated with VNPs, another reason for their appearance is that VNPs have been reported to help people reduce or quit smoking. People have reported that VNPs can help to relieve the desire to smoke and reduce cigarette consumption [94-97]. Research has indicated that the majority of VNP users use VNPs for either complete (79%) or partial replacement (17%) of tobacco cigarettes [98] (although numerous other studies have reported negative findings). A laboratory-based study was conducted to monitor cigarette consumption after using VNPs [99, 100]. The consumption results demonstrated VNPs delivered nicotine into the blood stream but the plasma nicotine level was not as high as regular cigarettes. This study also observed nicotine abstinence symptom suppression and found that VNPs reduced cigarette craving and increased satisfaction with nicotine abstinence. The most recent research compared the appeal of VNPs to conventional cigarettes and smoking cessation aids. It was found that VNPs had similar appeals to cigarettes, fewer side effects, and suited personal preference [101]. Furthermore, VNPs deliver nicotine faster than traditional NRT [102, 103], potentially increasing their appearance among users looking for a replacement product that more closely mirrors the experience of smoking. Other studies also hypothesised that besides delivering nicotine to the lungs, VNPs might provide a coping mechanism for conditioned smoking cues by replacing

some of the rituals associated with smoking gestures (for example, the hand-to-mouth action of smoking) as well. Thus, VNPs might assist in coping with cigarettes cravings [97, 100].

However, there is insufficient knowledge about how VNPs are used by smokers under real-world conditions, or whether these products actually help smokers to quit [104]. The evidence of VNPs relative profile to cigarette use, existing smoking cessation treatments, and the health risks, are still being explored. Such information is crucial to understanding how smokers use these devices and whether they may function as long-term cigarette substitutes. This knowledge could assist with regulation of the VNPs as well as examining the effects on individuals and at a public health level.

Furthermore, unlike NRT products, VNPs are largely unregulated and as a result manufacturing quality is inconsistent [105]. Wide variations exist among brands and even models within a brand. There is also variability across solutions intended for VNPs. Solutions with nicotine concentrations ranging from 0 to 36 mg/mL can be found for purchase over the internet, and with flavours that come in categories with labels such as tobacco (e.g, "classic," "sahara," "Cuban cigar"), menthol (e.g, "menthol ice," "peppermint patty"), dessert (e.g, "cotton candy," "bubble gum," "cheesecake"), and fruit (e.g, "apple," "raspberry," "watermelon") [88]. The actual nicotine concentrations may differ from the product labelling. Even in the labelled nicotine free (0 mg) liquids, trace amounts of nicotine have been detected [105]. Moreover, there is no standard label definition of nicotine levels in cartridge solutions. Some solutions are labelled with ambiguous descriptions of nicotine levels, such as "low," "medium," or "'high". Research evaluated the accuracy of nicotine labelling and the presence of nicotine impurities and degradation products (known as nicotine by product during the producing process) in 20 VNP liquid samples [106]. They found that nicotine

levels were 85-121% of what was labelled, while nicotine degradation products were present at levels of 0-4.4% [106]. With the latest models, VNP users can mix their own solutions to customise flavours and / or nicotine concentrations. Mixing solutions in a non-hygienic environment carries the risk of contamination, and failure to use proper safety clothing and procedures could lead to accidental nicotine poisoning [101]. The varied and uncertain content of nicotine in solution makes interpretation of the use of VNPs in clinical studies difficult. In other words, it will be difficult to evaluate the strength of nicotine that generates the effect of reduction when using VNPs as intervention tools in smoking studies.

2.3 Determination of smoking reduction

2.3.1 Biological markers determination

Smoking reduction is usually determined as a 50% reduction in cigarettes consumption [107, 108]. However, the exact harm associated with each cigarette is not consistent. Smokers can compensate for reduction by smoking each remaining cigarette more intensely. Thus, simply measuring the reduction of cigarette consumption is not a sufficient determinant of harm reduction. To better quantify smoking reduction, biological markers are used to determine the harm exposure. Biological markers can be used to measure nicotine and its metabolites in biological fluids, thereby assessing exposure to tobacco products.

After nicotine enters the body, it is extensively metabolised by a variety of liver enzymes and several metabolites have been reported in the literature [109] (Figure 2.1). Nicotine and its metabolites can be measured in blood, urine and saliva. Nicotine is metabolised by cytochrome P450 2A6 to cotinine, which is the main substance of nicotine metabolism. Cytochrome P450 enzyme is the main nicotine metabolising enzyme in humans [110]. About 66% of nicotine is metabolised to cotinine. Cotinine (around 75%) is further

metabolised to at least six secondary nicotine metabolites. The main metabolite is 3'-hydroxycotinine, which accounts for around 33-40% of total nicotine in urine (Figure 2.2) [111]. Glucuronidation is another pathway of nicotine metabolism, which occurs for both nicotine and its metabolites – cotinine and trans-3'-hydroxycotinine. Glucuronidation is catalysed by uridine 5'-diphospho-glucuronosyltransferase enzyme producing nicotine-N-β-glucuronide [112].

Nicotine also forms other chemicals during curing process (Figure 2.3). 4-(Methylnitrosamino)-1-(3-pyridyl)-1-1butanone (NNK), N'-nitrosonornicotine (NNN), N'-nitrosoanabasine (NAB), and N'-nitrosoanatabine (NAT) are formed by oxidation and nitrosation of nicotine. These nitrosamines are known as tobacco-specific nitrosamines, which are one group of carcinogens in tobacco products, and are also present in tobacco smoke [113]. NNK is extensively metabolised to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), which is also a potent pulmonary carcinogen. NNAL has a chiral center at the carbinol carbon, thus is formed as both (R)- and (S)-NNAL enantiomers, however, this reaction is enantioselective. (S)-NNAL is the major form (>90%) produced using lung cytosolic fractions, lung microsomes formed primarily (R)-NNAL (>95%). (S)-NNAL is more readily formed from NNK, it is also more readily glucuronidated and detoxified by the phase II UDP-glucuronosyltransferase (UGT) than (R)-NNAL in monkeys and probably in humans [114]. NNAL is further metabolised to glucuronides. Two isomers of NNAL-glucuronides, NNAL-O-glucuronide and NNAL-N-glucuronide, are detected in human urine. N-Glucuronidation, carried out by UGT 1A4 enzyme in human liver microsomes, has been implicated as an important mechanism of NNK detoxification pathway [115]. In addition, O-glucuronidation also carried out by UGT enzymes but different UGT subtypes to N-glucuronidation in liver micromes [116]. Although NNAL-N-glucuronides have been

reported to contribute substantially to total NNAL-glucuronides, both N- and O-glucuronidation are important in the NNK detoxification pathway. Hence, urinary total NNAL (free NNAL and its glucuronides) is a biomarker of NNK intake from tobacco, and directly links NNK exposure to lung cancer development in humans [117].

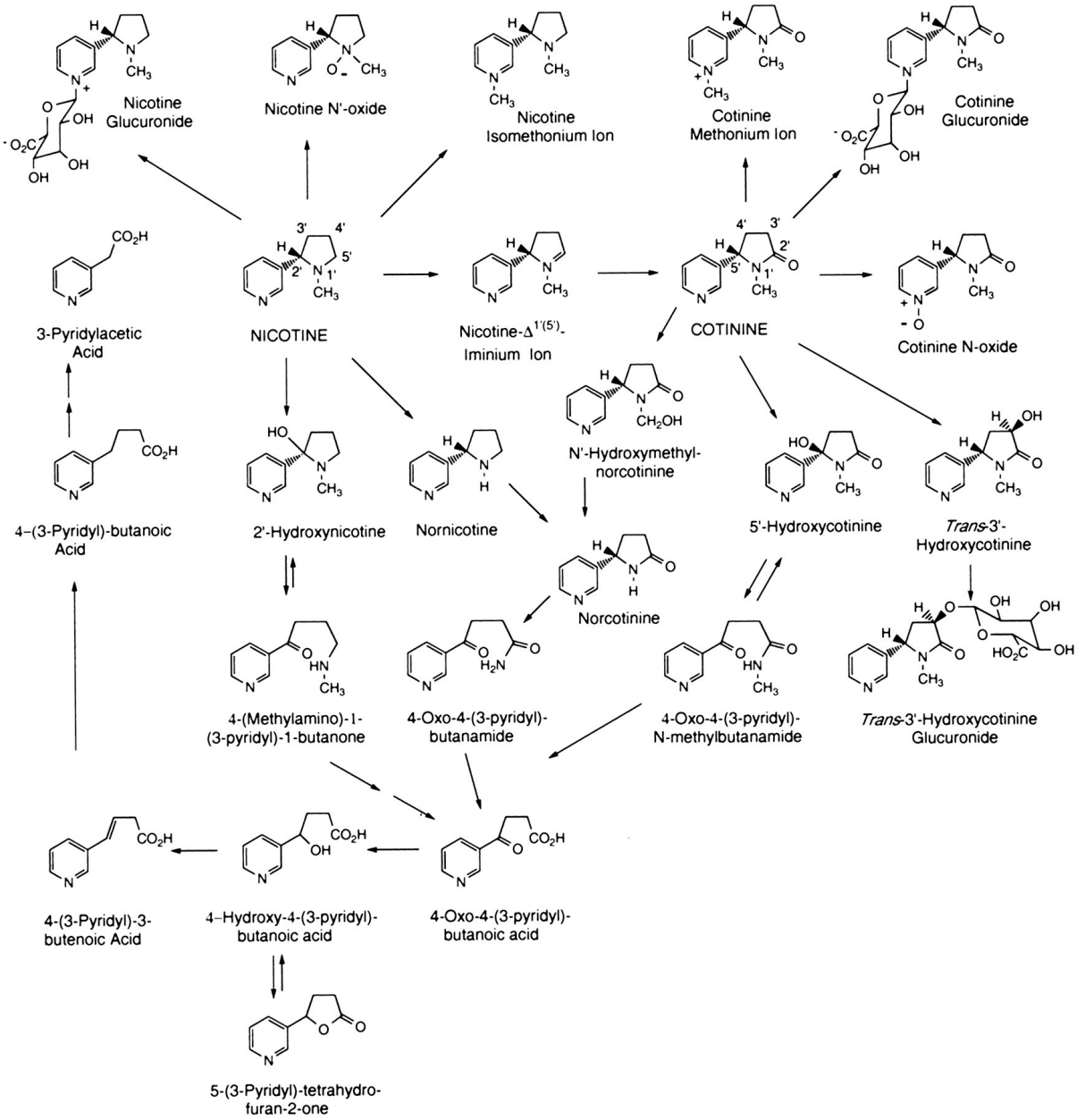

Figure 2.1 Nicotine metabolism pathway by liver [109]

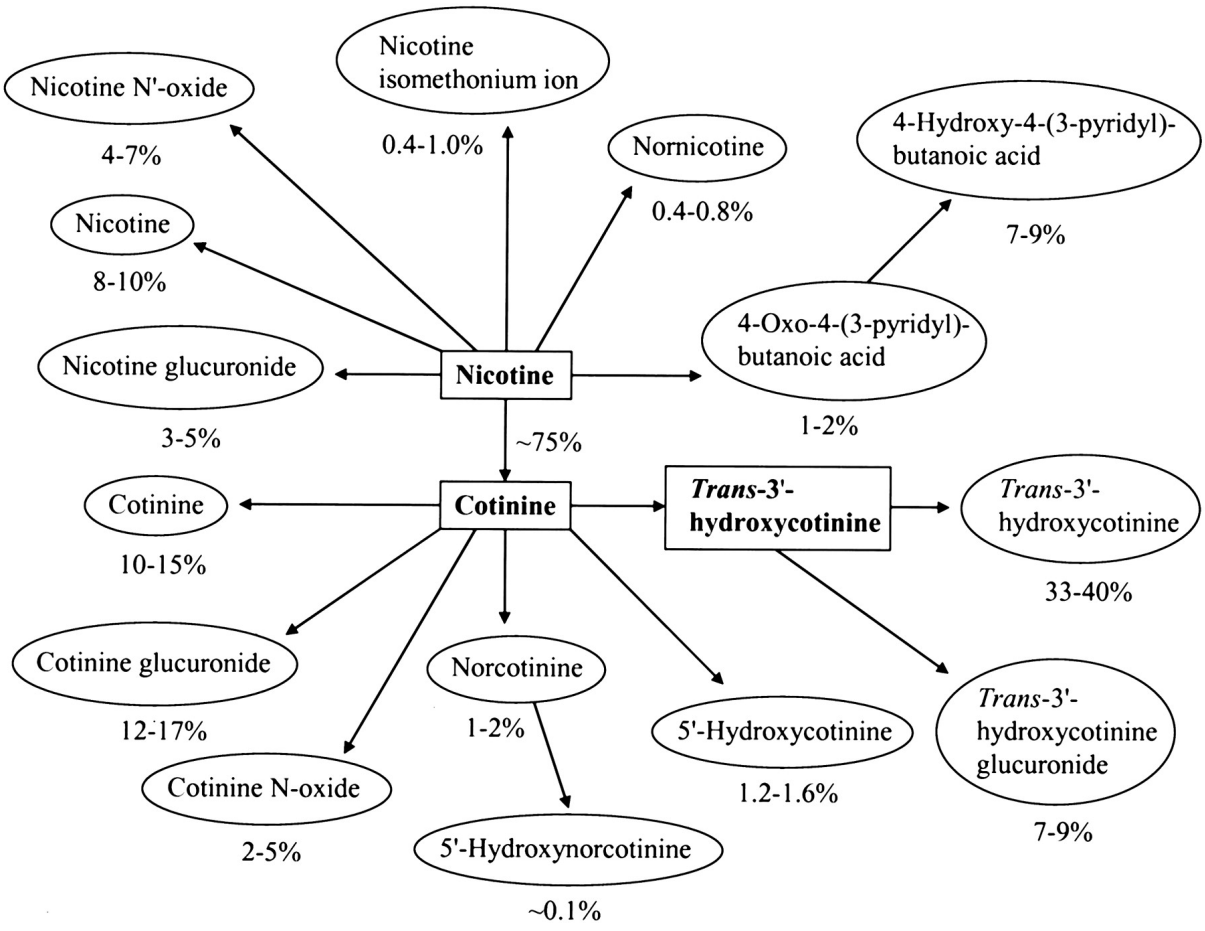

Figure 2.2 Estimates percentage of average extraction of nicotine metabolites in urine [118]

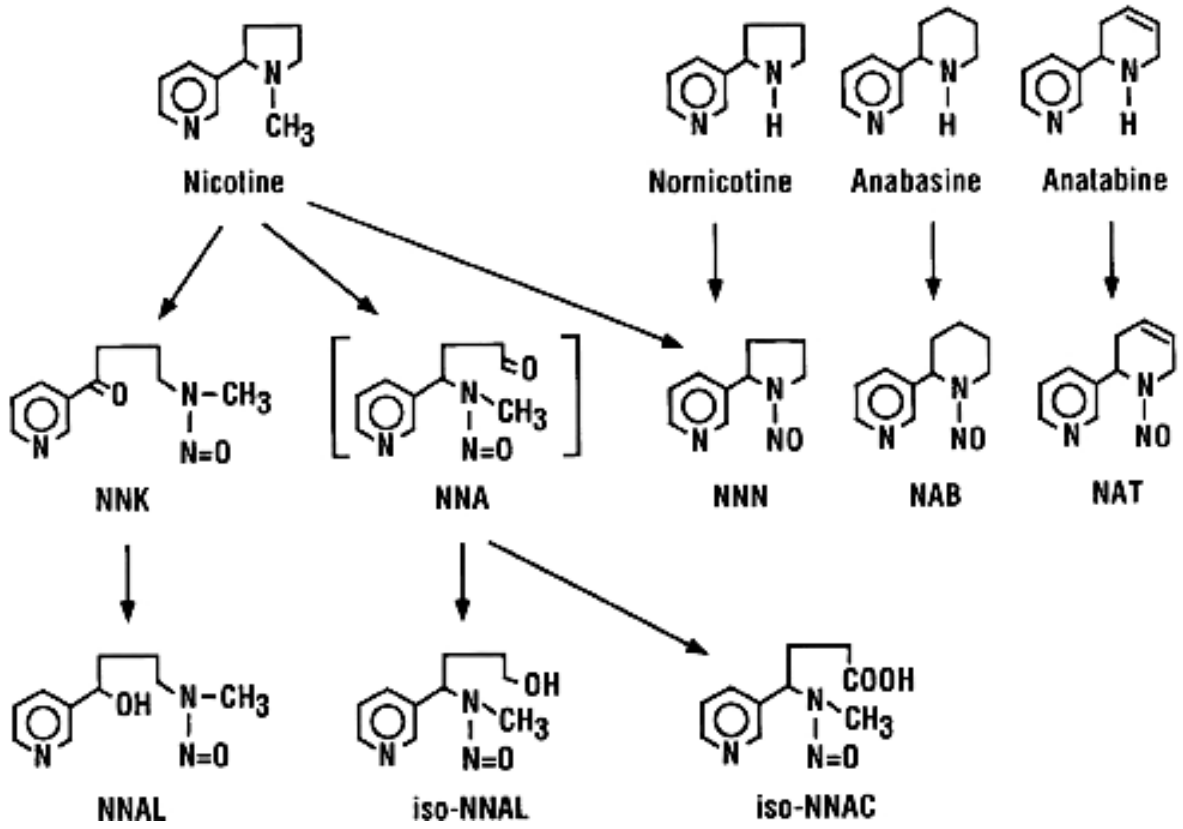

Figure 2.3 Structure of tobacco specific nitrosamines forms from nicotine during curing process [113, 119]

NNK is known as a tobacco-specific nitrosamine and is considered to be the most carcinogenic of the tobacco-specific nitrosamines. NNK is formed from nicotine and related compounds by a nitrosation reaction that occurs during the curing of tobacco. NNK is only present in tobacco products and tobacco smoke. It is assumed the uptake of NNK would be decreased with smoking reduction and cessation. NNK is metabolised to NNAL by cytochrome in P450 enzymes. The major metabolite, NNAL, is not present in tobacco products or tobacco smoke. In addition, NNK itself is not generally detected in the urine of laboratory animals treated with the compound, or in smokers' urine. Therefore, NNAL can be

used as a biomarker of NNK exposure from tobacco [120]. The long biological half-life of NNAL also can be used to determine the exposure of tobacco-related harm [121].

NNAL levels in urine are at trace levels (in range of ≤0.5 ng/mL) [122]. To determine NNAL levels, the most frequently utilised assay involves solid phase extraction (SPE) associated with liquid chromatography technique for analysis [117, 123]. In addition, a new trend of SPE technique, online SPE associate with liquid chromatography-tandem mass spectrometry (LC-MS/MS), has also been used to determine NNAL levels [124]. This technique is directly connected to the chromatographic analysis without the prior purification, which is less time consuming than using the normal SPE methods. Other related methods have been used for NNAL analysis, such as, high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) coupled with liquid-liquid extraction [125, 126]. In this case, the liquid-liquid extraction involved chemical derivatisation of NNAL, and the extraction took one day to complete, being a costly and time consuming process.

Because of the limitations of previous assays, there is an interest in developing a simple approach for NNAL determination. Ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) was chosen. Compared to normal HPLC, UPLC uses small particle-packed columns with a small diameter, operates with high pressures and obtains better resolution of peaks. UPLC has advantages in terms of analysis efficiency and time-saving [127]. UPLC has been used successfully to measure metabolites in biological fluid in smoking cessation studies previously [128, 129].

2.3.2 Nicotine content measurement in VNPs

To better quantify reduction, researchers have developed methods for assessing the constituents of nicotine substitutes as a method of measuring intake and exposure. To better understand VNPs (e-cigarettes), existing assays were mostly designed to analyse the levels of the main ingredients – nicotine – and its related compounds in e-cigarette liquids. For example, recent studies measured the nicotine and nicotine-related compounds in e-cigarette refill liquids using HPLC [106, 130]. These assays were restricted in application to a liquid cartridge, but there is a different cartridge type which is based on a fibrous material pad in the cartridge. Few assays have been applied to an e-cigarettes cartridge [131, 132]. Gas chromatography with the Thermionic Specific Detector (GC-TSD) [131] and HPLC-UV [132] have been used in one study. A major limitation in this study [131] was that quinolone was used as internal standard which has different chemical properties from nicotine. As a result, assay performance may not accurately represent the response of nicotine in the e-cigarette cartridge. Gold standard analytical procedure should generally use deuterated internal standards with mass spectrum based detection methods.

2.4 Research purpose

Tobacco harm reduction is designed to encourage smokers to gradually increase the use of treatment (e.g., NRT) and cut down cigarette consumption. Reduction might have an impact on increasing quit attempts and achieving successful cessation. Biological markers and the constituents of nicotine substituted products are used to measure tobacco intake and exposure. A variety of methods have been used to measure both biological markers of tobacco exposure and the constituents of nicotine products. However, there are limitations with existing methods to measure actual tobacco exposure. The research purpose is to 1) develop improved measures of the constituents of medical nicotine products, particularly in VNPs; 2) develop

measures of biological markers and apply these to assess smoking reduction; and 3) effectively use pre-quit treatments and accurately study smoking behaviour and its consequences.

Firstly, with the high interest in tobacco harm reduction, a variety of medical nicotine products have been utilised to assist harm reduction. However, if the nicotine content in the products is unknown or inconsistent, it is difficult to say that the products can be useful in assessing smoking reduction, particularly since the introduction of vaporised nicotine products (VNPs). With this question in mind, Chapter 3 of this thesis includes the development of an assay to determine the nicotine content in VNP products, and the application of this assay to VNP products to investigate whether the nicotine content is consistent with the label and consistent between product batches (Study 1). Knowing the nicotine content is of critical importance to allow a better interpretation of clinical outcomes while using these new products in future smoking reduction studies.

Secondly, reduced CPD is normally defined as the main determinant of smoking reduction. However, smoking behaviour can vary between individuals. Assessing the success of harm reduction by simply counting the CPD is not a sufficient measurement of harm reduction. Cigarette numbers used as the only physical determinant is not sufficient to monitor smoking reduction. Biological markers are normally used to assess smoking and nicotine intake. Current methods for NNAL determination involve complex sample preparation. An easier approach is required for testing NNAL levels in smokers' urine. Chapter 4 of this thesis contains the development of an assay for determining NNAL levels in human urine (Study 2), and Chapter 5 applies this NNAL assay to smokers' urine that has been collected from a smoking cessation study (Study 3).

Thirdly, a gradual reduction in smoking by giving a pre-loading treatment prior to the target quit day can help to increase the quit attempt by reducing the satisfaction of smoking, and improve the effectiveness of treatment [52]. Pre-quit nicotine patches have been utilised to give the pre-loading treatment 1-2 weeks prior to the target quit day in a previous cessation study [133]. Over the course of the pre-quit period, there was a reduction in satisfaction with smoking and in the number of self-reported cigarettes smoked, and that the reduced satisfaction might drive the smoking reduction and promote interest in quitting. Varenicline, has also been suggested to have similar efficacy as pre-quit patch treatment to reduce the satisfaction from smoking. However, the mechanism of reduction of satisfaction and smoking rate has not explicitly been tested before [57]. In order to gain a better understanding of smoking reduction, in Chapter 6 of this thesis, a clinical trial has been conducted to utilise two pre-quit treatments, nicotine patch and varenicline, to explore the effect of the two pre-quit treatments on reduction of daily smoking rate and satisfaction with smoking; and test the relationship between the satisfaction with smoking and smoking rate during the pre-quit period (Study 4). Monitoring such reductions may help to better tailor treatments.

The final two chapters of this thesis are a general discussion and conclusion regarding assays for determining smoking harm reduction, the determination of the constituents of nicotine substitute products, and biological markers of actual smoking harm exposure. These measures should assist the study of smoking behaviour and its consequences.

Chapter 3: Determination of Nicotine in Cartridge-based Electronic Cigarettes

Chapter 3

3.1 Background

Electronic cigarettes (e-cigarettes) as a new form of nicotine delivery device are evolving rapidly. However, due to lack of standards for manufacturing and regulations of e-cigarettes, there are concerns about the safety of their use. One of the main concerns is quality control during their manufacturing, especially the accuracy of labelling where nicotine content varies from manufacturer to manufacturer [10, 105, 131]. For instance, with many models, it is unknown whether nicotine content in the e-cigarette cartridge corresponds to the content claimed on the label. The properties of e-cigarettes are also important for producing vapour and delivering nicotine. Because there is lack of manufacturing standard for e-cigarette products, the design properties – for example battery power, airflow, pressure drop, etc. – vary among devices [134]. These factors influence the production of aerosol density from puff-to-puff, and subsequently influence the experience of using e-cigarettes and nicotine delivery. The variability of e-cigarette properties could result in the highly varied delivery of actual nicotine amounts in the vapour [105, 135, 136].

Only a few laboratory analytical studies have investigated the actual nicotine content in e-cigarette cartridges [105, 131]. One study has reported a method using methanol as an extraction solvent. This method used a platform shaker for 90 min extraction, then the extraction solution was analysed by high performance liquid chromatography (HPLC) [105]. While the authors reported 99% recovery of nicotine from the cartridge, the recovery was calculated simply based on a standard solution of nicotine, rather than extraction of nicotine from the actual sample matrix (the e-cigarette fibrous pad in this case). This is a major limitation in terms of analytical quality assurance, and inferior to the analytical approach taken in our work. In addition, this method prepared the standard solution of nicotine in 50% methanol in water and serially diluted with 10% acetonitrile in water, which was different to

the extraction solution (methanol). Differences in solvents used for extraction and standards can have a significant effect on instrument response (sensitivity). Furthermore, recovery estimates will also potentially vary significantly based on the types of cartridge materials used in e-cigarettes.

Therefore, there is an important need to develop and validate ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) technique for determination of nicotine content in e-cigarette matrices. The aim of the study was to develop an effective quantitative assay, and apply it to evaluate nicotine content in one specific type of e-cigarette product – fibrous pad based e-cigarette cartridges – using deuterated standards to obtain reliable recovery where blank cartridges are not available. It is of particular importance when interpreting the outcomes of studies designed to evaluate the safety of e-cigarettes, and / or their usefulness for smoking cessation, and / or smoking reduction, particularly given the rate at which new products are emerging.

3.2 Study design

The main ingredient in an e-cigarette is nicotine that is absorbed onto a fibrous material pad in the cartridge. An assay was designed for extracting nicotine from the fibrous pad and measuring the nicotine content. For investigating the extraction efficiency and assay validation, standard nicotine should be spiked onto a blank e-cigarette cartridge fibrous material pad. However, blank fibrous pads were not available for assay development because nicotine has been detected even in labelled nicotine free cartridges. Three approaches were used for the extraction. Firstly, an aliquot volume of nicotine-d_4 solution, used as an external standard, was spiked into the cartridge fibrous pads (n= 3) using a syringe and then the syringe was flushed twice with methanol. These samples were defined as the nicotine-d_4

group. Secondly, an aliquot of nicotine standard solution, that contained nicotine content equivalent to the content in the cartridge, was spiked into the cartridge pads (n= 3) by using the same syringe, then the syringe was flushed twice with methanol. In addition, nicotine-d_4 was spiked into the pads at the same amount as the nicotine-d_4 group. These samples were defined as nicotine standard addition group. Thirdly, the same process but an aliquot volume of methanol without nicotine was spiked into the cartridge pads (n= 3), which was defined as the control group. All the samples were placed in an Erlenmeyer flask and added to 50 mL of extraction solvent. The flasks were placed in the ultra-sound bath for a different time duration of extraction at ambient temperature. After the sonication, nicotine-d_4 solution was added as an internal standard in the extract of the control group. All the extraction solutions from three groups were further diluted with deionised water and analysed by UPLC-MS/MS.

3.3 Materials

3.3.1 Reagents

Nicotine standard (1.01 g/mL, 1 mL), purchased from Sigma Aldrich Co. (Saint Louis, Missouri, USA) was used for the preparation of standard solutions. Deuterated nicotine (nicotine-d_4) (100 μg/mL, in 1 mL Acetonitrile), was purchased from Cerilliant (Cerilliant Corporation, QLD, Australia), and used as an external standard. Methanol (50% v/v) in deionised water (methanol: MERCK KGaA, Darmstadt, Germany) and ethyl acetate (Sigma Aldrich CO., Saint Louis, Missouri, USA) were used as the extraction solvents. Trypan Blue solution (0.4%) (Sigma Aldrich Co., NSW, Australia) and Crystal Violet (0.1% w/v) (Oxid, West Heldleberg, VIC, Australia) were used to determine the penetration property of the fibrous pad.

3.3.2 E-cigarette cartridge samples

E-cigarettes (labelled as containing nicotine16 mg; Elusion New Zealand Holdings Ltd, New Zealand) were used. Ingredients were adsorbed onto a fibrous material pad in the e-cigarette cartridges. The ingredients were shown on the package, and included; nicotine, propylene glycol, glycerine, and tobacco flavour agent. Nicotine was classified under Schedule 7 substances of the Poisons Act 1971, regulation 74 of the Poisons Regulation 2008. We received authorisation from Vice-Chancellor of the University of Tasmania to purchase, store and use these products only for laboratory-based research.

3.3.3 Instrumentation

A Water Acquity® H-class UPLC system (Milford Massachusetts, USA) coupled to a Waters Xero® MS/MS using MassLynx™ software was used to detect and quantify the extracted nicotine solution. Chromatography was performed on a Water Acquity® UPLC BEH C_{18} column (2.1 × 100 mm × 1.7 micron particles). The laboratory equipment that were used during the experiments included an ultrasound bath (Bandelin Sonorex RK 100, Germany); vortex mixer type 37600 (Thermolyne Corporation, Dubuque, Iowa, USA); micropipette (Biohit, Germany).

3.3.4 UPLC solvent and analytical conditions

The UPLC solvent system consisted of mobile phase A (0.4% ammonia) and B (acetonitrile). The flow rate was 0.4 mL/min, with 100% A for 0.3 min, then a linear gradient to 30% A and 70% B at 4 min, before immediate re-equilibration to the initial condition for 3 min. The MS was operated in positive electrospray ionisation mode with multiple reaction monitoring (MRM). The ion source temperature was 150 °C, the desolvation gas was nitrogen at 1000 L/h, the desolvation temperature was 300 °C and the capillary voltage was 2.8 KV. The

MRM transitions monitored for quantitation were *m/z* 163.1→130.1 (nicotine), *m/z* 167.1→ 134.1 (nicotine-d_4).

3.3.5 Standard stock solution preparation

Nicotine standard (1.01 g/mL, 4.29 mg), liquid form at ambient temperature, was weighed and made up to 10 mL in volumetric flask with deionised water. A large volume of nicotine stock solution was also prepared by weighting 102.36 mg of nicotine and made up to 50 mL in volumetric flask with deionised water.

3.3.6 Calibration curve solution

The correlation coefficient (r^2) was used to estimate the linearity over a concentration range from 50 to 400 ng/mL for the calibration solutions of nicotine in methanol (50% v/v) in deionised water. The calibration solutions were prepared from the nicotine stock solution (4.29 mg/10 mL). Because the content of nicotine in the e-cigarettes was unknown, validation of the method was performed at two different levels of nicotine; low (200 ng/mL) and high (400 ng/mL). These values were equivalent to 10 mg and 20 mg of nicotine content in e-cigarette cartridges respectively, and were anticipated to cover the expected e-cigarette content range.

The dilution methods might be a factor that influences the linearity of calibration curve. We designed serial dilution and separated dilution of the standard solutions. With serial dilution, the nicotine standard solution was diluted from 400 ng/mL to 50 ng/mL. With separated dilution, the nicotine standard solution was diluted from 400 ng/mL to 200 ng/mL; 100 ng/mL; and 50 ng/mL separately. Ten microliters of nicotine-d_4 were added into each

concentration of nicotine solution. The mixed solution was analysed by UPLC-MS/MS to set up the standard calibration curve.

3.4 Extraction development

3.4.1 Extraction solvent effects

In order to investigate the appropriate solvent for extraction, we compared the chromatography response of nicotine standard in four solvents: methanol and ethyl acetate which were used as reference extraction solvents [105, 131], and another two solvents, 50% v/v methanol in deionised water and 100% deionised water. Nicotine (1 μg) was added in 50 mL of each solvent. The flasks were placed in the ultra-sound bath for 15 min at ambient temperature (20 to 25 °C). After sonication, the extract was further diluted with deionised water (1000-fold) before determining the nicotine content using UPLC-MS/MS.

3.4.2 Penetration property of cartridge fibrous pad

Nicotine-d_4 was investigated as an external standard by spiking it onto the surface of a cartridge fibrous pad and allowing it to penetrate into the matrix. The nicotine-d_4 was used to determine the extraction efficiency and measure the nicotine amount in the pad. However, it was unclear whether the nicotine was absorbed into or adsorbed onto the cartridge fibrous pad. The penetration property of the fibrous pad was therefore investigated. Two water soluble dye were used, Trypan Blue solution (0.4%) and Crystal Violet (0.1% w/v). Ten microliters of each dye were spiked onto the sliced e-cigarette cartridge fibrous pad, allowing the solution to penetrate into the pad and photographed after 5, 10, and 15 min of penetration.

3.4.3 Nicotine-d_4 external standardisation

As mentioned before, nicotine-d_4 was used as an external standard by spiking onto the surface of the e-cigarette cartridge fibrous pad. In order to obtain a reliable recovery estimate, another requirement was that the external standard must be fully spiked into the sample cartridge penetrating the matrix. Two nicotine-d_4 spiking methods were investigated; an HPLC syringe technique versus a micropipette technique. Ten microliters of nicotine-d_4 (1 µg) were spiked onto the surface of the fibrous material using a 25 µl glass HPLC syringe with a stainless steel blunt tip or micropipette with disposable plastic tip. The solution was allowed to soak into the fibrous pad for 10 min. The pads then underwent the extraction process.

3.4.4 Extraction solvent

Ten microliters of nicotine-d_4 (1 µg) were spiked onto the surface of the e-cigarette cartridge fibrous pad (nicotine-d_4 group, n= 4); ten microliters of nicotine-d_4 (1 µg) and ten microliters of nicotine standard (16 mg) were spiked onto the surface of the fibrous pad (nicotine standard addition group, n= 4); and ten microliters of methanol were spiked on the fibrous pad (control group, n= 4); and allowed to penetrate into the fibrous pad for 10 min. The pads were then placed in Erlenmeyer flasks and 50 mL of methanol, ethyl acetate, 50% v/v methanol in deionised water, and 100% deionised water were added to different flasks to investigate the effect of extraction solvent. The flasks were placed in the ultra-sound bath for 15 min at ambient temperature. After sonication, the same amount of nicotine-d_4 was added into control group samples, and all the extracts were further diluted with deionised water. Given that nicotine-d_4 (external standard) and nicotine analyte were different in concentration, two separate dilutions of the extraction solutions were used; 50-fold dilution for nicotine-d_4 and 1000-fold for nicotine determinations respectively. Both diluted extraction solutions were then analysed using UPLC-MS/MS.

3.4.5 Extraction time

Extraction time was investigated as a determinant of extractive capacity. Cartridge fibrous pads (n= 3) were removed from the e-cigarette cartridge chamber and extracted as outlined above for different time periods. Samples were extracted using ultra-sound bath (15 min), ultra-sound bath (30 min), shaker (60 min) and shaker (120 min) at ambient temperature. The extract was further diluted with deionised water before analysed by UPLC-MS/MS.

3.4.6 Application of assay on e-cigarettes batches

E-cigarette cartridge fibrous material was removed from the cartridge chamber using a pair of tweezers. Ten microliters of nicotine-d_4 (1 μg) were spiked onto the fibrous material using a micropipette and allowed to penetrate into the material for 10 min. The fibrous material was then placed in an Erlenmeyer flask with 50 mL of the appropriate extraction solvent (50% v/v methanol in deionised water). The flask was placed in the ultra-sound bath for 15 min at ambient temperature. After sonication, the extract was further diluted with deionised water. Given that nicotine-d_4 and nicotine analyte were orders of magnitude different in concentration, two separate dilutions of the extraction solutions were used; 50-fold dilution for nicotine-d_4 and 1000-fold for nicotine determinations respectively. Both diluted extraction solutions were then analysed using UPLC-MS/MS.

3.4.7 Assay performance

Acceptance criteria for the assay were defined as intra-day accuracy and precision less than 5% at these levels. Within-batch intra-day precision was evaluated by repeated injections (n= 5) of nicotine standard solution (400 ng/mL) throughout the course of one day. Intra-day accuracy was calculated from the calibration curve at the 200 ng/mL and 400 ng/mL solution.

Because blank e-cigarette cartridges were not available, the recovery of nicotine was calculated based on the nicotine-d_4 content extracted from the fibrous material compared to a nicotine-d_4 standard diluted to a concentration equivalent to 100% theoretical recovery from the e-cigarettes. Recovery was calculated both intra-day (n= 5) and inter-day (n= 6). Limit of detection (LoD) and lower limit of quantification (LLoQ) were determined from the signal-to-noise ratio of replicate injections (n= 5) at the 50 ng/mL level for nicotine, with LoD defined as a signal-to-noise ratio of 3 and LLoQ as a signal-to-noise ratio of 10 [137]. Carry over was assessed by injecting blank deionised water following injection of standards containing 400 ng/mL nicotine; selectivity was assessed as analyte response in blank deionised water (n= 5).

3.5 Results and discussion

3.5.1 Method development

This method was designed to extract and measure the content of nicotine present in e-cigarette cartridges consisting of a fibrous pad. The assay is particularly useful where blank cartridges are not available. Results in our laboratory and others have shown that even nicotine free labelled cartridges may contain nicotine [105]. Previous studies have shown that nicotine content did not match the label claim with some brands [105, 131]. But these assays have had minimal validation with respect to recovery of analytes from the cartridge pad. This current assay used a known content of nicotine-d_4 as a standard spiked onto the fibrous cartridge pad material, which was then compared with the nicotine-d_4 standard using external standardisation to calculate the recovery of nicotine-d_4 from the pad. During the development of the assay, different extraction solvents were investigated. The chromatogram peak area of nicotine in three approach groups was investigated in four extraction solvents. Compared to deionised water (100%), the mean peak area of two replicates of nicotine in methanol

solution was 136 ± 0.01%, 50% v/v methanol in deionised water was 76 ± 2.7%, and ethyl acetate solution was 69 ± 5.1%. The results have shown that the choice of solvent influences nicotine recovery and response with up to 50% of variation between solvents.

It was found that care was required with regard to the exact amount (~1 μg) of nicotine-d_4 spiked onto the fibrous pad material. The penetration of the external standard solution from the surface was confirmed visually. The two water soluble dyes that could be seen absorbed into the pad material with no observable difference between 5, 10 and 15 min (Figure 3.1). A glass HPLC syringe was used to spike nicotine-d_4 onto the pad, and a micropipette was used to spike nicotine-d_4 as well. These two methods resulted in different recoveries of nicotine-d_4; ~50-60% by using the HPLC syringe technique and 81% by using the micropipette technique. The recovery of nicotine-d_4 was lower with the syringe than with the micropipette, possibly due to capillary action (Figure 3.2).

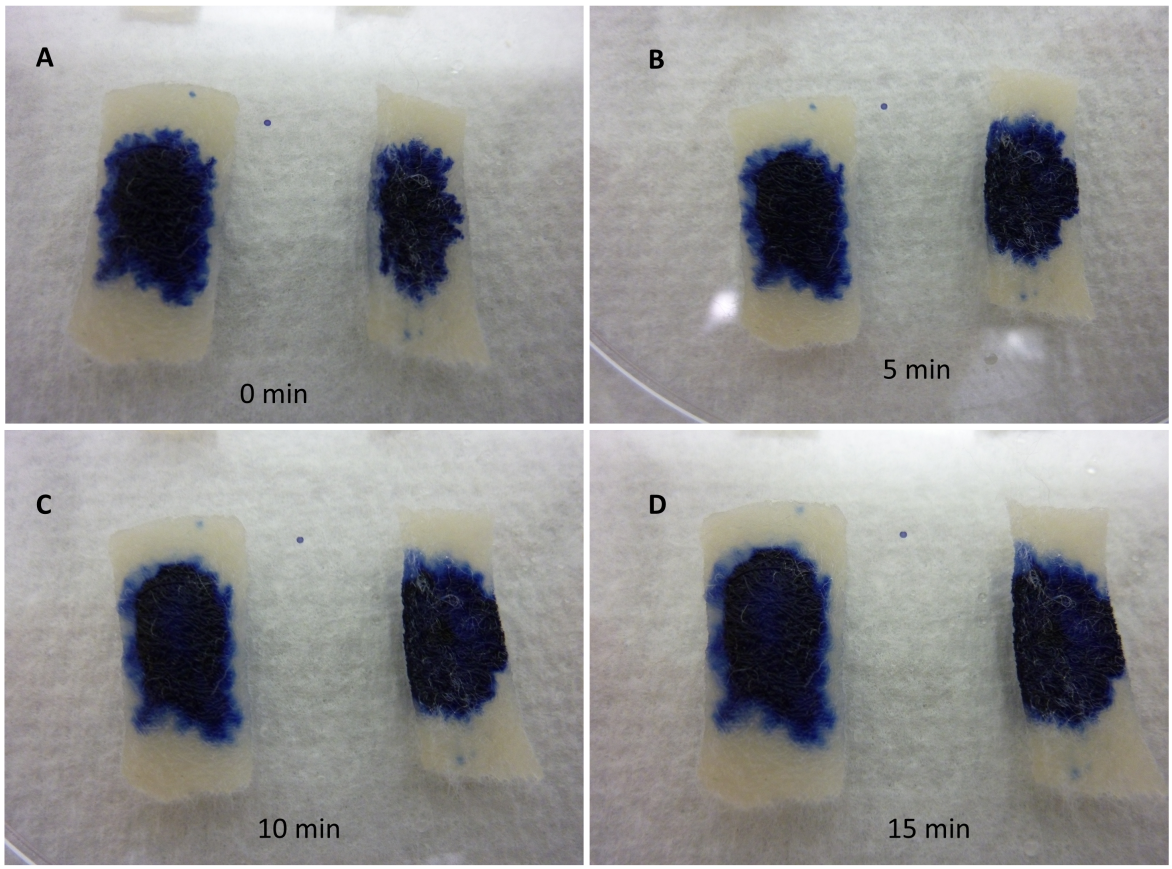

Figure 3.1 Ten microliters of two water-soluble dyes, Trypan Blue solution (0.4%) (left side fibrous pad) and crystal violet (0.1% w/v) (right side fibrous pad), were spiked onto the sliced e-cigarette cartridge fibrous pad, allowing the solution to penetrate the pad. A= 0 min after spiking; B= 5 min after penetration; C= 10 min after penetration; D= 15 min after penetration

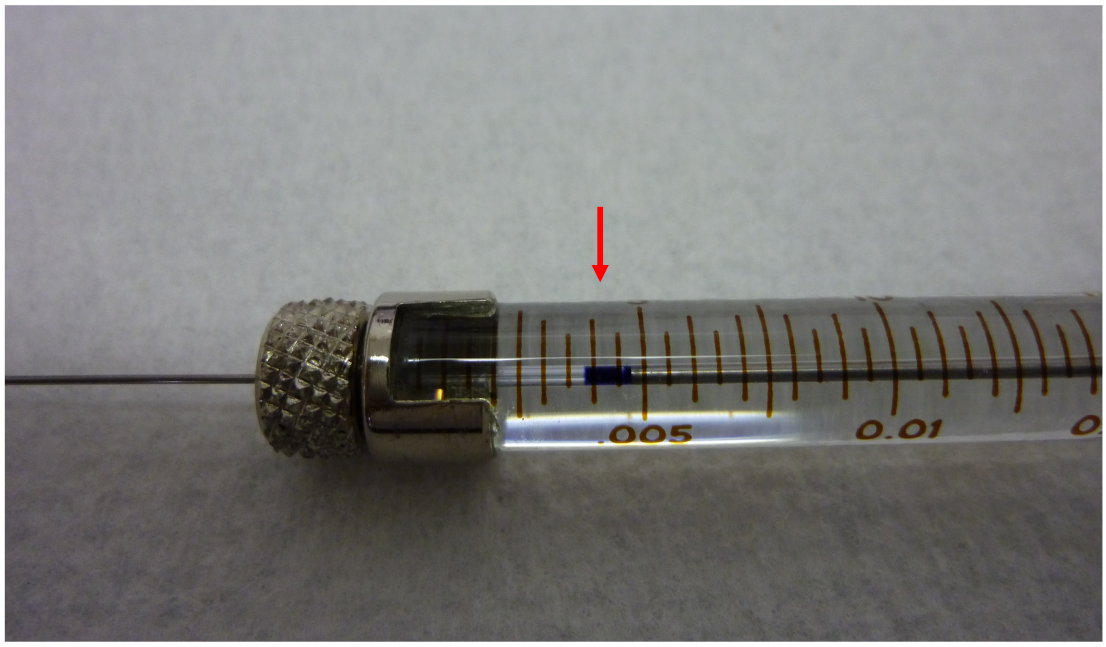

Figure 3.2 The dye solution remained in the syringe when the HPLC syringe was used to spike nicotine-d_4 into the cartridge fibrous pad

In addition, extraction time would be expected to be a determinant of extractive capacity. Therefore, three extraction time periods were investigated, 15 min, 30 min, and 60 min. The recovery of nicotine-d_4 was 86% with 15 min ultra-sound bath, 79% with 30 min ultra-sound bath and 83% and 84% with 60 and 120 min on a shaker. The recovery results showed that there was a little difference between these so the shortest time was chosen for this assay.

A significant barrier in the development of this assay was the large difference between amounts of nicotine-d_4 standard that could be used economically and the nicotine concentrations in the e-cigarette fibrous core (approximately 16,000-fold). It was not feasible to dilute the extraction solvent to a level whereby both analytes were in the calibration range

of the instrument. Nicotine-d_4 is relatively expensive and it is not economical to spike at concentrations equivalent to nicotine. Therefore, the extraction solution was diluted separately, which necessitated two different analyses for each sample; 1000-fold dilution for nicotine and 50-fold dilution for nicotine-d_4, and similarly for the external standard calibration solutions. Using the nicotine-d_4 spiked onto the cartridge pad, and under our extraction conditions, we consistently demonstrated greater than 80% recovery.

3.5.2 Assay performance

The chromatograms for nicotine and nicotine-d_4 in solvent extracted from an e-cigarette cartridge are shown in Figure 3.3. Nicotine and nicotine-d_4 eluted at 3.85 and 3.82 min respectively. Nicotine standard is a viscous liquid, which might cause a deviation between the theoretical amount and the actual measured amount when utilising micro-pipette to measure and transfer volumes. For instance, if preparing nicotine standard solution with a theoretical nicotine amount of 400 µg, due to the viscosity of nicotine, the actual measured amount might less than 400 µg. Hence, nicotine standard was weighed using Mettler Toledo balance (0.001 g) to prepare the nicotine standard stock solution. The nicotine standard calibration curve was linear over the range of 10 ng/mL to 400 ng/mL with an r^2= 0.9998 (Figure 3.4). Intra-day accuracy was 0.7% and 0.5% at 200 ng/mL and 400 ng/mL levels respectively. Intra-precision [relative standard deviation (RSD%)] was 3.1% and was consistent when repeated on a second day (3.6%). The LoD was 0.04 ng/mL and LLoQ was 0.14 ng/mL. The recovery of nicotine-d_4 extracted from the cartridge pad using 50% v/v methanol in water was 81.5% ± 3.7 and 82.4% ± 1.3 for intra-day and inter-day respectively (mean ± SD). In the nicotine standard addition group, there was a corresponding increase in nicotine peak area compared with nicotine-d_4 group and control group.

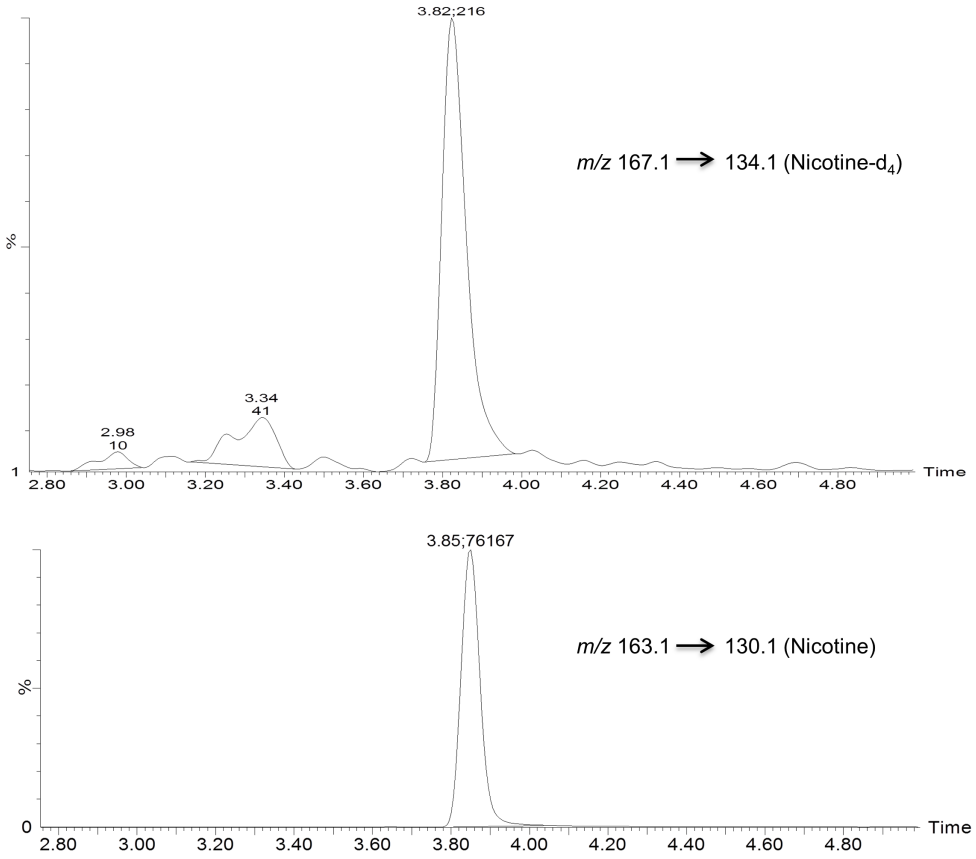

Figure 3.3 Sample chromatograms showing nicotine-d_4 external standard (top) and nicotine (bottom) recovered from e-cigarettes cartridge fibrous pad

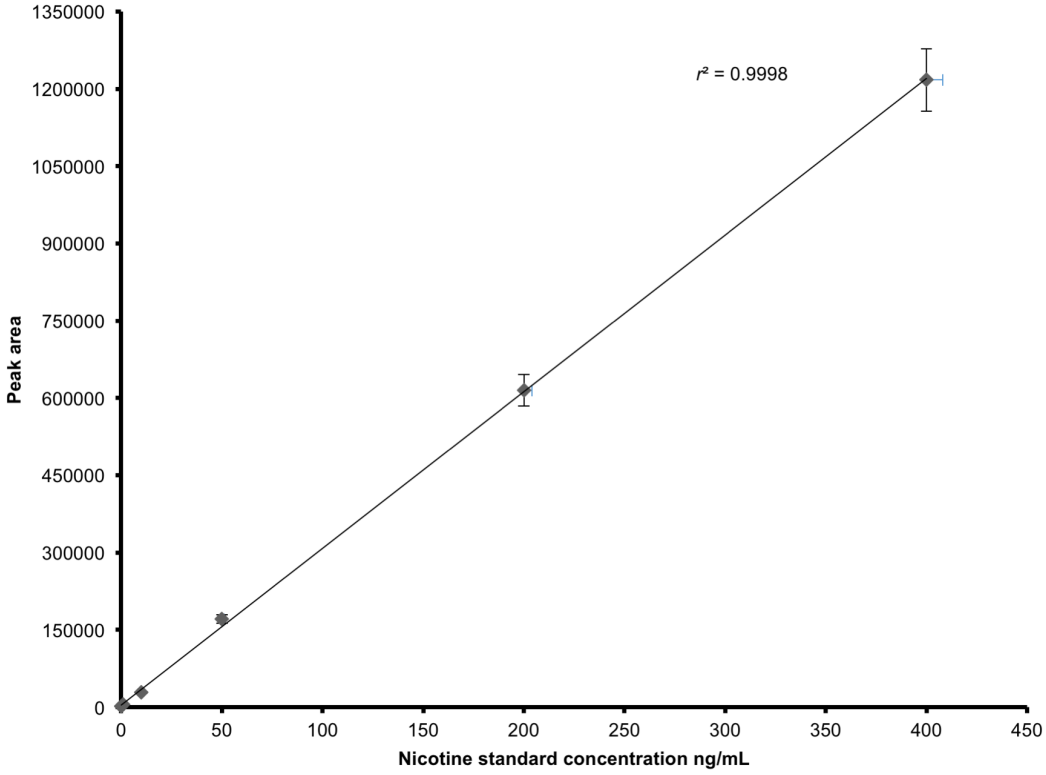

Figure 3.4 The nicotine standard calibration curve was linear over the range of 10 ng/mL to 400 ng/mL with an r^2= 0.9998. Error bar represents the standard deviation

3.5.3 Nicotine content in e-cigarette batches

The developed and validated assay was applied to e-cigarette batches (same brand of e-cigarette as noted in 2.2.2). The recovery of nicotine-d_4 extracted from the cartridge pad was 81.5% ± 3.7 (mean ± SD). Taking into account recovery, nicotine content in the e-cigarette cartridge samples, 11.9 ± 1.3 mg (mean ± SD), and the variation of nicotine content between cartridges within batch as well as between batches was not significant (Figure 3.5). With this method, significant differences were found between labelled and actual content of nicotine in e-cigarette cartridges. The nicotine content of all the cartridge samples was ~25% lower than the labelled content (16 mg). This result indicated that the nicotine content information on the

product package provided by the manufacturer may mislead customers and that direct comparison with e-cigarettes is difficult, although it is well-known that smokers will titrate the dose obtained from smoking [99]. How e-cigarettes are used can impact on the actual amount of nicotine absorbed [138].

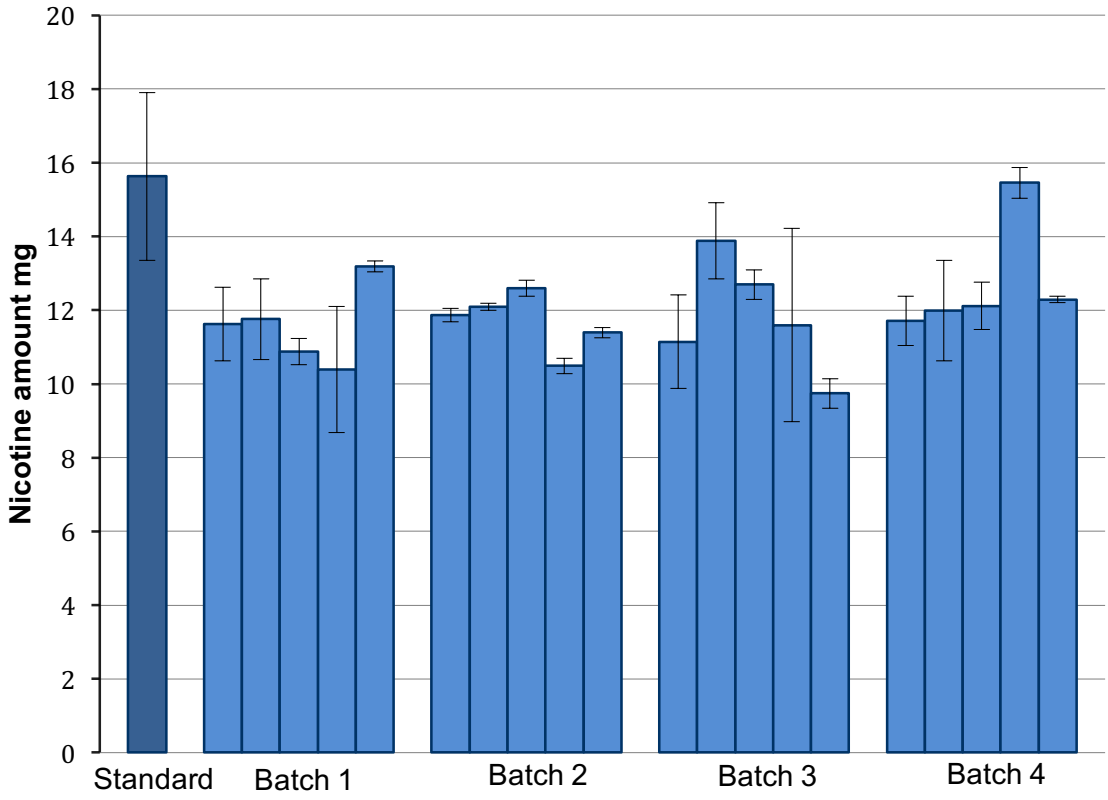

Figure 3.5 Nicotine content in one brand of e-cigarette cartridges (all depicted as mean ± 95% confidence intervals). A nicotine standard (16 mg) was shown on the left (n= 8 replicates) and compared with four packs of the same brand of e-cigarette and from each pack a random sample of five e-cigarette cartridges were analysed (n= 3 replicates)

3.6 Conclusion

An efficient assay was developed to measure the nicotine content in e-cigarette cartridges containing fibrous pads in propylene glycol and glycerine solvents where blank e-cigarettes are not available. The content of nicotine in the e-cigarette cartridges can be effectively determined by UPLC-MS/MS. Application of this assay demonstrates that, at least with the e-cigarettes we tested, the amount of nicotine stated on the label varied substantially from that observed analytically. The accurate determination of the quantity of nicotine in e-cigarettes has important implications for both consumer safety and the further study of these devices.

Chapter 4: Determination of 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) in Urine

Chapter 4

4.1 Background

Thousands of chemicals are generated during curing tobacco, and most of them are toxic and carcinogenic [139]. Tobacco-specific nitrosamines (TSNAs) are one group of carcinogens in tobacco products and are also presented in tobacco smoke [113]. Among the tobacco-specific nitrosamines, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is one of the most prevalent of these compounds presented in both unburned tobacco and cigarette smoke. It is a remarkably effective lung carcinogen in laboratory animals, inducing lung tumours in rats, mice, and hamsters independent of the route of administration [113, 140]. NNK, which is generated from nicotine by oxidation and nitrosation during the curing process, has been classified as a human carcinogen (Group I) by the International Agency for Research on Cancer [139]. 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), the major metabolite of NNK, is also a potent pulmonary carcinogen [120]. NNAL is further metabolised to N- and O-glucuronide conjugates, which are excreted in urine together with free NNAL [115, 140]. Total NNAL (free + glucuronide) is not presented in cigarette smoke, hence it is considered an effective biomarker of NNK uptake [120]. The long biologic half-life of NNAL, which is 10-18 days, can also be used to determine the exposure of tobacco-relate harm [121].

NNK is formed from nicotine and related compounds by a nitrosation reaction that occurs during the curing of tobacco. Hence, the uptake of NNK would be decreased with smoking cessation. NNAL levels in urine are typically in range of less than 0.5 ng/mL, and vary from individual to individual [122]. Measuring low levels of NNAL in a urine matrix is a significant analytical challenge. Urine matrix might interfere the extraction of low level content, typically like NNAL levels at 0.5 ng/mL. In previously reported liquid chromatographic assays, NNAL measurement utilised a solid phase extraction (SPE)

technique to concentrate naturally occurring NNAL levels prior its analysis [124, 141]. However, the reported SPE methods have been complex, with multiple steps, including two SPE extractions with different stationary phase and solvents. An easy to approach assay is needed to determine the NNAL level in smokers' urine.

Recent new extraction techniques, including dispersive liquid-liquid micro-extraction (DLLME), dispersive liquid-liquid micro-extraction based on the solidification of floating organic droplets (DLLME-SFO), are simple, quick, inexpensive, and efficient techniques with a high enrichment factor for sample preparation (Table 4.1). These techniques are particularly applicable for trace level analytes in a sample, such as in urine matrices. DLLME and DLLME-SFO assays are generally established on a ternary component solvent system, in which an appropriate disperser solvent is introduced to help the dispersion of an organic extraction solvent into an aqueous sample and improve the extraction efficacy [142-144]. The use of DLLME or DLLME-SFO to analyse NNAL in urine samples has not been reported so far. Apart from these relatively new techniques, liquid-liquid extraction is a traditional and frequently used technique in separation and analytical chemistry. UPLC-MS/MS is a sensitive and selective assay that we developed previously for the determination and quantification of the two major nicotine metabolites, cotinine and trans 3'-hydroxycotinine, in smokers' urine [128]. UPLC-MS/MS assay has not been applied to measuring NNAL levels in previous research studies. Therefore, the objective of this study was to develop a simple rapid assay by using UPLC-MS/MS combined with an efficient but easier sample preparation methodology to determine NNAL levels in urine samples. Additionally, this assay applied to smokers' urine samples to investigate both free and total NNAL levels as well as to assess tobacco exposure.

Table 4.1 DLLME and DLLME-SFO assay summary

	Requirements	Extraction solvents	Disperser solvents	Technique	Advantage	Reference
DLLE	low solubility of extraction solvent in aqueous sample	1,1,1-trichloroethane	methanol	HPLC-UV	minimal consumption of extraction solvent (micro volume)	[142, 145]
	density is greater than water	tetrachloroethane	acetone	GC-MS	short extraction time	
		dichloromethane	acetonitrile		high enrichment factor	
		chlorobenzene	ethanol			
		chloroform	2-propanol			
DLLE-SFO	low solubility of extraction solvent in aqueous sample	undecanol	acetone	HPLC-MS		[143, 146-148]
	density is lower than water	dodecanol	methanol			
	high melting point	hexadecane	acetonitrile			
		toluene	ethanol			

4.2 Study design

Five extraction assays, including DLLME, DLLME-SFO, derivatisation of NNAL, SPE and liquid-liquid extraction, were designed for NNAL extraction. After the extraction, the NNAL extract was evaporated with nitrogen gas and reconstituted in deionised water for UPLC-MS/MS analysis. Assay validation was processed using the spiked NNAL standard and deuterated NNAL (NNAL-d_3) blank urine samples. The extraction efficacy would be directly affected by the selection of extraction and disperser solvents during each extraction in the assay development. The extraction efficacy could also be determined by ultra-sound bath sonication, as well as different extraction durations. The recovery of NNAL was used to determine whether the assay was suitable for NNAL extraction.

4.3 Materials

4.3.1 Reagents

NNAL standard (10 mg) and deuterated NNAL (NNAL-d_3) (1 mg), were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada), and used for the preparation of standard solutions and internal standard respectively. HPLC-grade methanol (Merck KGaA, Darmstadt, Germany) was used to prepare the standard solutions. Ethanol (Merck Pty Ltd, VIC, Australia), acetone (Wesfarmers CSBP Ltd, WA, Australia) and acetonitrile (Sigma-Aldrich, NSW, Australia) were used as the disperser solvents. Dichloromethane (DCM) (Waters Associated, Milford, MA, USA), 1-undecanol (Sigma-Aldrich, MO, USA), hexane (Fisher Chemical, Pittsburgh, USA), toluene (Merck KGaA, Darmstadt, Germany), and HPLC-grade ethyl acetate (Merck KGaA, Darmstadt, Germany) were used as extraction solvents. Trifluoroacetic acid (TFA) (0.1% in water (v/v), pH 3, Ajax Chemical, NSW, Austraia) and ammonia solution (28%, pH 10, Ajax Chemical, NSW, Australia) were prepared to adjust urinary pH.

4.3.2 Instrumentation and apparatus

The UPLC-MS/MS instrument and the laboratory equipment have been described in 3.3.3. Reacti-Vap Evaporator with Reacti-therm I heating module (ThermoFisher scientific, Victoria, Australia) was used to control the evaporation temperature and evaporate the extracted organic phase.

4.3.3 UPLC solvent and analytical conditions

The UPLC solvent system and column condition were the same as described in 3.3.4. Injection volume was 20 μL. The MS was operated in positive electrospray ionisation mode with MRM. The ion source temperature was 130 °C, desolvation gas was nitrogen at 950 L/h, desolvation temperature was 450 °C, and the capillary voltage was 2.8 kV. Cone voltage was optimised for each MRM transition at 24 V. Dwell time for each transition was 78 msec.

4.4 Methodology

4.4.1 Preparation of calibration solution

A standard stock solution of NNAL (200 μg/mL) was prepared by dissolving 10 mg of NNAL standard in 50 mL of methanol in a volumetric flask. Similarly, deuterated NNAL (NNAL-d_3) stock solution (100 μg/mL) was prepared by dissolving 1 mg of NNAL-d_3 in 10 mL of methanol in a volumetric flask. The stock solutions were stored in a refrigerator at 4 °C for up to six months. NNAL working standard solution (5 ng/mL) and NNAL-d_3 internal standard solution (5 ng/mL) were freshly prepared from the stock solutions and used within two days. Calibration standard solutions were prepared by spiking NNAL working standard solution in blank urine over an ascending concentration range of 1 to 400 pg/mL, and spiked with 100 pg/mL of internal standard.

4.4.2 UPLC-MS/MS performance

The MS was operated in positive electrospray ionisation mode with MRM. NNAL working standard solution (5 ng/mL) and NNAL-d_3 internal standard solution (5 ng/mL) spiked in a blank urine was prepared for the full scan of NNAL and NNAL-d_3 mass range. The retention time of NNAL and NNALd3 was monitored by using UPLC.

4.4.3 Assay performance validation

Correlation coefficient (r^2) was used to estimate the linearity of NNAL in the range from 1 to 400 pg/mL. Acceptance criteria for the assay were defined as the intra-day accuracy and precision values below an RSD% of 15% at the 5, 20 and 100 pg/mL of standard samples, which represented low, medium and high levels of NNAL in smoker's urine. The recovery of NNAL was calculated based on the NNAL-d_3 concentration in urine samples compared to a NNAL-d_3 standard prepared to a concentration equivalent to 100% theoretical recovery from the blank urine samples. The LoD and LLoQ were determined from the signal-to-noise ratio of replicate injections (n= 6) at 5 pg/mL of a standard sample with LoD defined as S/N= 3 and LLoQ as S/N= 10 [149]. Carry over was assessed by injecting a blank deionised water sample following injection of a standard containing100 pg/mL of NNAL. Selectivity was assessed as analyte response in blank deionised water (n= 3). Freeze/thaw stability was determined by freezing (-20 °C) and thawing (ambient temperature) standard samples (5 pg/mL, 20 pg/mL, and 100 pg/mL, n= 6) for three cycles [150]. Bench top stability was assessed over 8 hours by leaving the standard samples (5 pg/mL, 20 pg/mL and 100 pg/mL, n= 6) on the bench top at ambient temperature (20 to 25 °C). Sample storage stability was investigated using the subjects' urine at the NNAL levels of 50 pg/mL, 120 pg/mL, and 300 pg/mL (n= 6) measured at baseline and after storage in a freezer (-20 °C) for a duration of one month.

4.5 Extraction development

4.5.1 DLLME assay development

Blank urine, collected from a non-smoker without any passive exposure to tobacco smoke in the previous 48 hours, was adjusted to pH 10 using ammonia solution. An aliquot (1 mL) was spiked with 40 µL of NNAL standard solution at concentration of 500 pg/mL (prepared from NNAL working standard solution at 5 ng/mL) and 20 µL of internal standard solution (5 ng/mL) equivalent to 20 pg/mL of NNAL and 100 pg/mL of NNAL-d_3 in urine respectively. Disperser solvent (300 µL; methanol, ethanol, or acetone) containing 50 µL of DCM (extraction solvent) was rapidly added into the urine solution using a micropipette. The sample tube was gently shaken for 1 min by hand to combine the organic solvents and aqueous solution. Then the capped test tube was put in an ultra-sound bath for 10 min. After sonication, a miscible solution with fine droplets was formed. The solution was centrifuge at 5000 rpm for 5 min and the organic phase containing analytes was separated on the bottom of the aqueous phase. The organic phase was transferred with a micro pipette into a glass vial and evaporated to near dryness using nitrogen gas for 25 min. The analyte was reconstituted with 100 µL of deionised water before analysing NNAL levels using UPLC-MS/MS.

4.5.2 DLLME based on solidification of floating organic drop (DLLME-SFO) assay development

Based on the concept of DLLME, 1-undecanol was used as an extraction solvent in the DLLME-SOF assay and acetonitrile was used as a disperser solvent. Similarly, blank urine was spiked with 40 µL of NNAL standard solution (500 pg/mL) and 20 µL of internal standard solution (5 ng/mL) equivalent to 20 pg/mL of NNAL and 100 pg/mL of NNAL-d_3 in urine respectively. Acetonitrile (300 µL) containing 50 µL of 1-undecanol was added into the urine solution. The tube was gently shaken for 1 minute by hand and kept in an ultra-sound bath for 10 min. Miscible solution with fine droplets combined analyte was formed after

sonication. The solution was centrifuged at 5000 rpm for 5 min; the supernatant organic phase was separated on the top of the aqueous phase. Then the test tube was placed in an ice bath for a few minutes to solidify the organic phase. The solidified organic phase was transferred into a new test tube and thawed at ambient temperature automatically. Acetonitrile (100 μL) was added into the organic solution to assist the nitrogen gas induced evaporation. In addition, different evaporation temperatures (25 °C, 32 °C, and 45 °C) and different evaporation durations (15 min, 25 min, and even up to 40 min) were attempted.

4.5.3 Derivatisation of NNAL

Blank urine sample was centrifuged at 5000 rpm for 5 min and hexane was then added to remove non-polar interferences in urine. An aliquot of urine sample (1 mL) was spiked with standard and internal standard solutions and mixed well. Ethanol-pyridine (300 μL, 4:1 v/v), a disperser solvent, was added in the sample, and ethyl chlorofomate (20 μL) was added as derivation solvent. After vortexing for 3 min, diethyl ether (1 mL) was added and the solution was gently shaken at 175 rpm (Shaker, Paton Scientific, Australia) for 15 min to pause the derivation reaction. Then the solution was centrifuged at 2000 *g* for 10 min. The supernatant phase (ethereal phase) was transferred into a glass vial and evaporated with nitrogen gas at 25 °C to near dryness. The analyte was then reconstituted in 100 μL of deionised water and transferred into an insert for NNAL analysis.

4.5.4 Solid-phase extraction (SPE)

Solid phase extraction cartridges (OASIS HLB cartridge, Waters, NSW, Australia) were conditioned with two 1 mL aliquots of acetonitrile, and washed with two 1 mL aliquots of deionised water. The conditioned cartridges were not allowed to dry out before the addition of the sample. An aliquot of blank urine sample (1 mL), spiked with standard (40 μL,

500 pg/mL), was added into the cartridge and passed through the cartridge, one mL of deionised water was used to wash the cartridge. After the aqueous phase was washed out of the cartridge, two aliquots (0.75 mL) of acetonitrile were added to elute the analytes from the cartridge and the elution was collected in 1.5 mL Eppendorf® tube. The elution was centrifuged at 5000 rpm for 5 min and transferred into a glass vial and evaporated with nitrogen gas at 25 °C to near dryness. The analytes were reconstituted with deionised water (80 µL) and spiked with internal standard (20 µL, 5 ng/mL) for NNAL analysis.

4.5.5 Liquid-liquid extraction

Blank urine was adjusted to pH 7, 10, or 12 using ammonia buffer. The samples were then spiked with 40 µL of working standard solution (5 ng/mL) and 20 µL of internal standard solution (5 ng/mL). Extraction solvent (ethyl acetate, hexane, or toluene) and disperser solvent (acetonitrile, 1 mL) were added to urine samples of each urinary pH, and with different urine/extraction solvent volume ratios (v/v). The tube containing spiked urine and solvents was shaken for 1 min by hand and vortexed for 3 min to ensure proper mixing of urine and solvents. Then the solution was kept for 5 min at ambient temperature to allow the separation of the solvent phase and aqueous phase, and then all the samples were centrifuged at 2000 *g* for 8 min. The supernatant organic phase was transferred into a glass vial. A duplicated extraction process was applied to all the samples, aimed to determine whether a repeat extraction procedure can improve the extraction efficacy. The supernatant organic phase was evaporated using nitrogen gas for 15 min to near dryness, then reconstituted with 100 µL of the deionised water before analysing for NNAL levels using UPLC-MS/MS.

4.6 Results and discussion

The developed extraction assays and recovery of NNAL in urine samples are summarised in Table 4.2.

4.6.1 UPLC-MS/MS performance

MS was set to scan a specific mass range of analytes. The full scan analysis of mass can assist to select the appropriate ion for quantification and qualification of analytes. The MS was operated in positive electrospray ionisation mode with MRM. At the beginning, with the selected MRM transition, *m/z* 210.1→ 149.1 (NNAL) and *m/z* 213.1→ 149.1 (NNAL-d_3), the chromatogram peak area of both NNAL and NNAL-d_3 was embedded with the background noise signals, which prevented use for quantitation, possibly due to the urinary matrix effects. Therefore, different MRM transitions were tested to find the optimal MRM transition for quantitation of NNAL. The appropriate MRM transitions monitored for NNAL quantitation were *m/z* 210.1→180.1 (NNAL), *m/z* 213.1→183.1 (NNAL-d_3). The chromatograms for NNAL and NNAL-d_3, extracted from blank urine samples, are shown in Figure 4.1. NNAL and NNAL-d_3 eluted at 3.62 and 3.61 min respectively. The final UPLC-MS/MS assay demonstrated satisfactory performance, meeting the acceptance criteria.

Table 4.2 NNAL extraction assay development and recovery of NNAL from urine samples

	Extraction solvent	Disperser solvent	Derivation solvent	Extraction method	Time (min)	Recovery %	Limitation
DLLME	DCM	ethanol	-	ultra-sound bath sonication	10	25	**l**ow recovery of NNAL
DLLME-SFO	1-undecanol	acetonitrile	-	ultra-sound bath sonication	10	-	1-undecanol cannot be evaporated
Derivation of NNAL	diethyl ether	ethanol-pyridine	ethyl chlorofomate	orbital shaker	15	-	by-product of derivatisation reaction forme**d**
SPE	acetonitrile	-	-	SPE cartridge	-	-	NNAL chromatogram peaks embedded with background noise signals
Liquid-liquid extraction	ethyl acetate	-	-	vortex shaker	3	65	

Note: samples for assay development were prepared by spiking 20 pg/mL of NNAL standard and 100 pg/mL of NNAL-d_3 into the blank urine; urinary pH 7 was adjusted with ammonia solution.

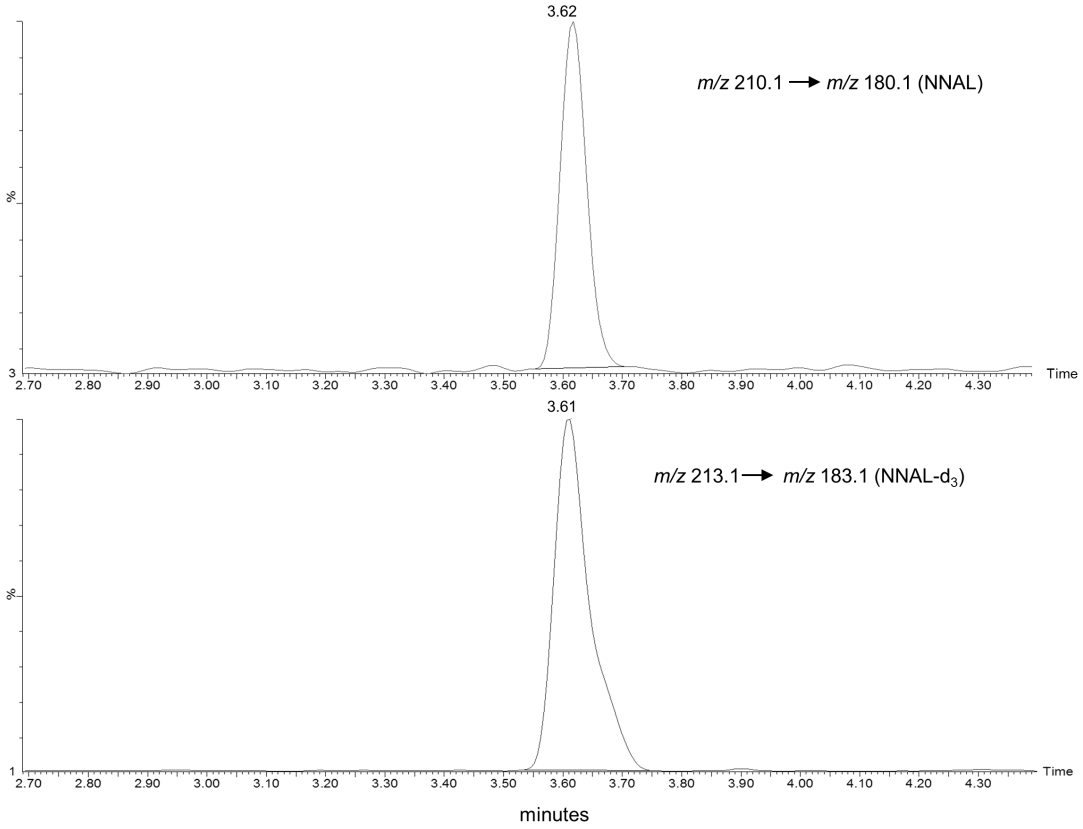

Figure 4.1 Representative UPLC-MS/MS chromatograms of NNAL and NNAL-d_3 in blank urine sample with a concentration of NNAL of 20 pg/mL

4.6.2 Urinary pH

NNAL has two calculated pKas, 13.5 and 5.4 [calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02, referenced from Scifinder]. In high pH or low pH solution, NNAL is mostly in the ionised state. The recovery of NNAL at urinary pH 7 was greater than at both higher urinary pH 10 and pH 12. Therefore, all the urine samples were adjusted to pH 7 for assay development.

4.6.3 DLLME assay performance

In general, there are several requirements to perform satisfactory extraction of an analyte using DLLME. Firstly, the extraction solvent should have low solubility in the aqueous phase and a greater density than water to facilitate phase separation by centrifugation. DCM met the requirements of an extraction solvent in the DLLME assay. The second requirement of DLLME is that the disperser solvent should be miscible with both the extraction solvent and the aqueous phase. The disperser solvent is used to assist the dispersion of an organic extraction solvent into an aqueous sample and to further obtain a high efficient extraction. Therefore, methanol, ethanol, and acetone were used for this purpose. As shown in Figure 4.2, a miscible solution was formed with both ethanol and acetone. In particular, ethanol formed a finely miscible solution. The formation of the miscible solution was due to the co-solvency of the disperser solvent with the extraction solvent and aqueous phase. Ethanol introduced a greater dispersion of DCM into aqueous phase than acetone. In contrast, methanol did not form a miscible solution. This may have been due to the hydrophilic properties of methanol that cannot be miscible with the aqueous phase. Therefore, ethanol was chosen as a disperser solvent. Ultra-sound bath sonication was used to improve extraction efficiency in DLLME assay as previously reported [151]. Different extraction times were applied (3 min, 5 min, and 10 min). A fine miscible solution was formed with 10 min ultra-sound bath sonication. Hence, DCM and ethanol were used with ultra-sound bath sonication to extract spiked NNAL from blank urine samples. However, the recovery of NNAL was low, only about ~25%, which was not efficient, especially given the low biological levels.

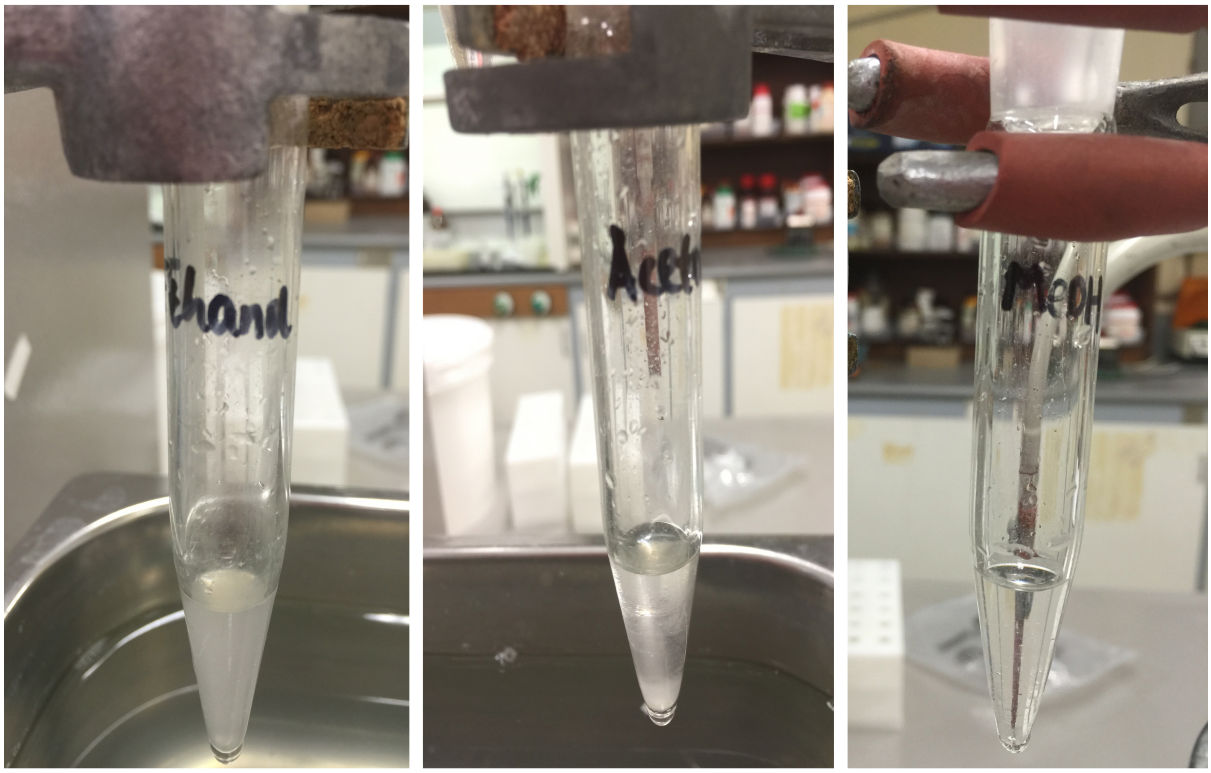

Figure 4.2 DLLME assay development. DCM was used as an extraction solvent; ethanol (left-side), acetone (middle) or methanol (right-side) was used as a disperser solvent. After 10 min sonication, miscible solutions were formed with ethanol and acetone but not with methanol

4.6.4 DLLME-SFO assay performance

A solution was required to overcome the issue of low recovery of NNAL in the DLLME sample preparation. Generally, organic solvents used in DLLME are toxic and limited to carcinogenic chlorinated solvents, such as 1,1,1-trichloroethane and tetrachloroethane. The extraction phase normally settles to the bottom of the centrifuge tube after centrifugation because of the higher density of chlorinated solvent than water. As an alternative, the concept of DLLME based on solidification of a floating organic droplet (SFO) has been developed [143, 147, 148]. Solidification of the floating organic droplet means that the extraction

solvent should have a melting point close to or below room temperature and the density must be lower than that of water. 1-Undecanol, a long-chained fatty alcohol that has a melting point at 11 °C and a lower density than water, will float on the top of the aqueous phase, solidify below 11 °C and melt at ambient temperature. Theoretically, 1-undecanol phase would be easier to transfer into a glass vial compared to DCM, which required a glass needle to transfer it from the bottom of the centrifuge tube. Thus, we developed an DLLME-SFO assay to extract NNAL from urine samples by using 1-undecanol as extraction solvent.

One problematic issue for 1-undecanol is that it is highly lipophilic with a boiling point of 243 °C, and therefore difficult to evaporate it directly with nitrogen gas. Mixing 1-undecanol with other lipophilic and hydrophilic solvents was used to assist the evaporation process. Acetonitrile, toluene, hexane, ethyl acetate, DCM, acetone, and methanol were added to the 1-undecanol containing extracted NNAL to identify which one of the solvents could assist the evaporation of 1-undecanol. Unfortunately, even the most lipophilic solvent – hexane – did not assist with the evaporation of 1-undecanol. The high temperature and long evaporation duration had the potential to lose the analytes, either through chemical decomposition or evaporative loss. The UPLC column conditions were also not suitable for analysis by directly injecting 1-undecanol as the extraction solvent. In previously reported study, UPLC column used for direct injection of 1-undecanol analysis was Agilent Tc C_{18} column (4.6 × 150 × 5 micron particles) and the mobile phase was methanol and water [143]. The UPLC column used in our assay was BEH C_{18} column (2.1 × 100 mm × 1.7 micron particles), which was narrower than the reported UPLC column. Furthermore, the mobile phase used in our assay was consisted of 0.4% ammonia and acetonitrile, which was more lipophilic than the one used in the reported study (methanol and water). The UPLC column conditions were suitable for all the chemical and biological analysis in our project, and it was

deemed not advisable to change the column conditions. Therefore, the two new extraction methods, DLLME and DLLME-SFO, were not suitable for NNAL analysis using our analytical conditions.

4.6.5 Derivatisation of NNAL

Derivatisation techniques using ethyl chloroformate to form alkoxycarbonyl alky esters have been used in urine matrices. In theory, NNAL would be amenable to this reaction through the butanol functionality. Ethylchloroformate techniques have been successfully applied to serotonin and bisphenol A derivatisation previously [152]. In addition, the ethylchloroformate derivatives are often much more sensitive to analysis by UPLC-MS/MS than the native analytes. Hence, we developed an ethylcholroformate derivatisation technique to determine NNAL level in urine. There was a spectrum signal observed at *m/z* 399, however, the signal was not an NNAL derivative, but possibly a by-product of the derivatisation reaction. Therefore, the derivatisation technique was also not suitable for NNAL analysis.

4.6.6 SPE assay performance

In previously reported NNAL assays, SPE assay has often been utilised to extract NNAL from urine samples [124, 141]. The extraction procedures were complex, including using multiple extraction solvents and multiple types of SPE columns in one study. Compared to those reported assays, our assay aimed to simplify the extraction procedure, only using one extraction solvent (acetonitrile) and one type of SPE cartridge. However, the matrix interference from urine resulted in the NNAL signals being embedded with the chromatographic background signals. The simplified SPE was not suitable for NNAL extraction in urine samples.

4.6.7 Liquid-liquid extraction assay performance

Finally, a simple liquid-liquid extraction assay was developed, based on the recovery of NNAL from spiked blank urine samples, by adjusting urinary pH, extraction solvent and urine/extraction solvent volume ratio (Table 4.3). There was no difference in recovery with or without disperser solvent. It was found that the recovery of NNAL at urinary pH 7 was greater than at pH 10 and 12, due to the pKas of NNAL. Recovery also increased with larger extraction volumes of urine (2 mL) and extraction solvent (v/v, 1:1). Duplicate extractions also improved the recovery of NNAL from urine samples as expected, due to an increase in extractive capacity. In addition, different centrifuge durations were applied (5 min, 8 min, and 10 min). The results showed that clear separation was obtained with 8 min centrifugation.

The NNAL standard calibration curve was linear over the range of 1 pg/mL to 400 pg/mL with an r^2= 0.9996 (Figure 4.3). Intra-day accuracy was 3.7%, 3.7% and 3.6% and precision was 11.3%, 5.1% and 5.3% at 5 pg/mL, 20 pg/mL and 100 pg/mL levels respectively. The LoD was 0.95 pg/mL and LLoQ was 3.2 pg/mL. Freeze/thaw stability results were within 15% and bench top accuracy was within 10% at 5 pg/mL, 20 pg/mL and 100 pg/mL respectively. Loss after one month of sample storage was 8-15%. Carry over and selectivity were lower than LoD and less than 5% of LLoQ.

Table 4.3 Recovery of NNAL from spiked urine samples during liquid-liquid extraction development

	Recovery %	
	Mean	SD
pH (ethyl acetate; v: v= 1:1)		
7	50	21
10	20	0.23
12	10	0.16
Extraction solvent (pH 7; v: v= 1:1)		
Ethyl acetate	55	12
Hexane	-	
Toluene	-	
Volume ratio [a] (pH 7; ethyl acetate)		
1:1	28	0.90
1:1×2 [b]	64	16
2:2	65	15

Note: 20 pg/mL of NNAL standard and 100 pg/mL of NNAL-d_3 was spiked into the blank urine; urinary pH was adjusted with ammonia solution; [a] volume ratio= urine/extraction solvent (v/v); [b]= duplicate extraction.

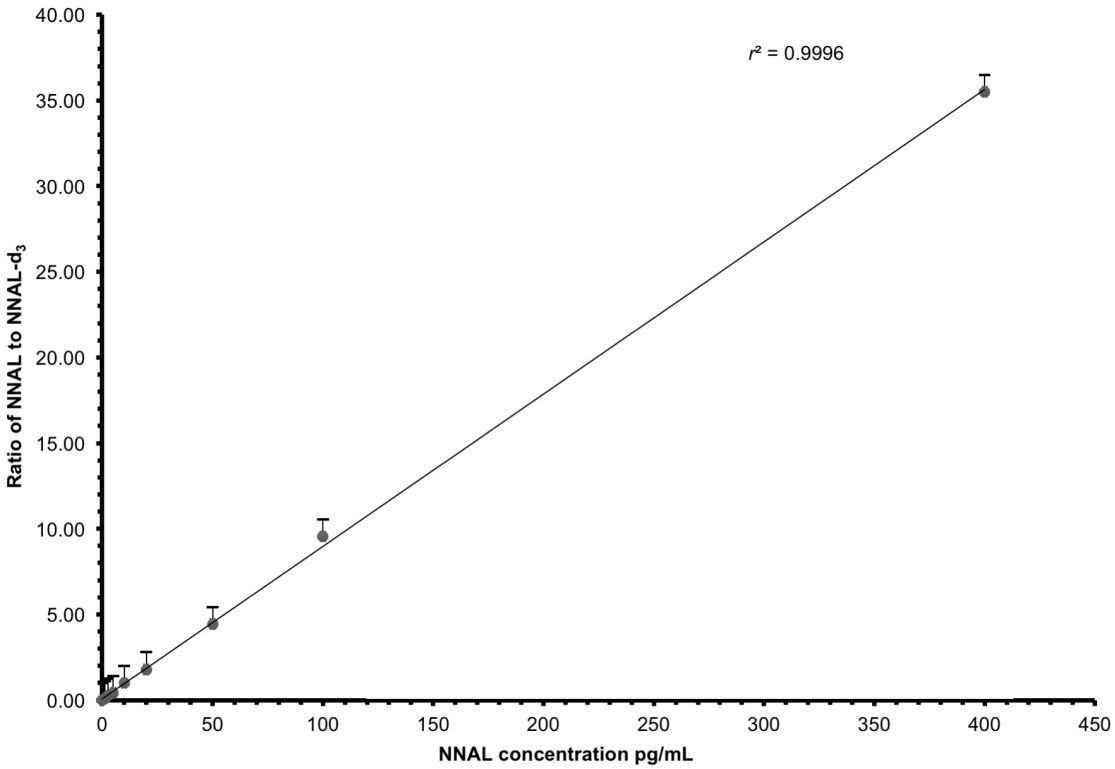

Figure 4.3 The NNAL standard calibration curve was linear over the range of 1 pg/mL to 400 pg/mL with an r^2= 0.9996. Error bar represent the standard deviation

This liquid-liquid assay is an improvement over existing SPE-based methods in that it is substantially less complicated while maintaining adequate sensitivity. The main extraction procedures are summarised in Table 4.4. The simple but sensitive NNAL assay we developed will be useful for studying the effectiveness of tobacco harm reduction strategies.

4.7 Conclusion

Based on a comparison between different extraction assays, liquid-liquid extraction with UPLC-MS/MS measurement is a convenient and sensitive approach to measuring low levels of NNAL in urine as a biological marker of NNK exposure. This method of determination can be used to assess tobacco-related exposure and the effectiveness of smoking reduction harm minimisation strategies.

Table 4.4 The procedure of liquid-liquid extraction assay compared with existing SPE-based assay for NNAL analysis

	Solvents	Facilities	Main procedures	Reference
SPE	methylene chloride	Chem Elut column	sample was eluted from Chem Elut column with methylene chloride	[123]
	toluene	Molecularly Imprinted Polymer (MIP) column	HCl was added into sample eluant	
	toluene-methylene chloride (9:1)	nitrogen gas evaporation	NaOH and phosphate buffer were added to neutralise HCl	
	hydrogen chloride (HCl)	HPLC-MS	MIP column was washed with toluene and toluene-methylene chloride (9:1)	
	sodium hydroxide (NaOH)		sample was eluted from MIP column	
	phosphate buffer (pH 6.4)		sample was dried by nitrogen gas and reconstitute in water	
Liquid-liquid extraction	ethyl acetate	vortex shaker	sample's urinary pH adjusted with ammonia solution	
	ammonia solution	nitrogen gas evaporation	ethyl acetate was added into sample	
		UPLC-MS/MS	shaked by hand for 1 min and vortexing for 3 min	
			organic phase containing analyte was evaporated with nitrogen gas and reconstituted in water	

Note: The precision and accuracy of liquid-liquid extraction assay met the assay validation acceptance data.

Chapter 5: Application of Assay for 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) in Urine for the Assessment of Tobacco-related Harm

Chapter 5

5.1 Background

Smoking has been widely known to be associated with adverse health effects. Cigarette smoking is the leading cause of preventable mortality and morbidity. Millions of people worldwide die from tobacco-related diseases each year [1].

While complete cessation of smoking is the preferred outcome, increasingly researchers and policy makers are exploring harm minimisation approaches, for example, smoking reduction using alternate forms of nicotine delivery to curb tobacco-related harms. A key argument supporting harm minimisation strategies is that by reducing cigarette smoking, we reduce exposure to tobacco toxins and, hence, subsequent tobacco-related morbidity and mortality [69]. The reduction can be determined by monitoring smoking patterns, such as cigarettes per day, withdrawal severity, and craving for cigarettes. Furthermore, an evaluation of such strategies necessitates measuring the level of hazardous tobacco-related chemicals present in biological samples, such as plasma and urine.

As introduced in Chapter 4, NNK is a known lung carcinogen presented in both unburned tobacco and cigarette smoke [121, 139]. And its major metabolite NNAL is considered an effective biomarker for exposure to tobacco-related harm [121]. It has also been proposed that the ratio of NNAL-glucuronide to total NNAL could be a potential biomarker [153] with rapid metabolism to the glucuronide inferring faster detoxification [154]. However, the importance of this ratio remains unclear, with different findings between studies and ethnic groups [124, 126, 154, 155]. Proper validation of analytical methods determining total (free + conjugated) NNAL remains difficult. These methods rely on enzymatic cleavage of the NNAL-glucuronide to yield the aglycone and then measuring the total NNAL after hydrolysis. This approach can lead to loss of accuracy and precision [156].

Chapter 5

A simple but sensitive bioassay of NNAL using liquid-liquid extraction and UPLC-MS/MS was developed (in Chapter 4) and applied to smokers' urine to assess tobacco exposure, investigating both free and total NNAL levels.

5.2 Materials

5.2.1 Reagents

The NNAL standard, NNAL-d_3 and extraction solvents were the same as described in section 4.3.1. *β*-Glucuronidase (type IX-A, *Escherichia coli*) and phosphate buffered saline tablets were purchased from Sigma-Aldrich (NSW, Australia).

5.2.2 UPLC Instrumentation and analytical condition

UPLC-MS/MS system analytical conditions were the same as described in section 3.3.3 and 3.3.4. The MRM transitions monitored for quantitation were *m/z* 210.1→180.1 (NNAL), *m/z* 213.1→183.1 (NNAL-d_3). Cone voltage was optimised for each MRM transition at 24 V. Dwell time for each transition was 78 msec.

5.2.3 Preparation of calibration solutions

A standard stock solution of NNAL (200 μg/mL) was prepared by dissolving 10 mg of NNAL standard in 50 mL of methanol in a volumetric flask. Similarly, NNAL-d_3 stock solution (100 μg/mL) was prepared by dissolving 1 mg of NNAL-d_3 in 10 mL of methanol in a volumetric flask. The stock solutions were stored in a refrigerator at 4 °C. NNAL working standard solution (5 ng/mL) and NNAL-d_3 internal standard solution (5 ng/mL) were freshly prepared from the stock solutions by dilution with deionised water. Calibration standard solutions were prepared by spiking NNAL working standard solution in blank urine over an

ascending concentration range of 1 to 400 pg/mL, and spiked with 100 pg/mL of internal standard.

5.2.4 Assay performance

Assay performance validation of accuracy, precision, LoD, LLoQ and sample stability was prepared in blank urine and processed as described in section 4.4.3.

5.3 Application among community samples of cigarette smokers

Urine samples (n= 48 samples) were collected from current smokers (n= 24) as part of a larger smoking cessation study. Participants were current smokers who were willing to use either varenicline (0.5 mg and 1 mg, Champix®, Pfizer Australia Pty Ltd) or nicotine patch (21 mg/24 hr, Nicabate®, GlaxoSmithKline Australia Pty Ltd) as part of a quit attempt. Participants provided a urine sample at baseline (prior to starting their quit attempt and during ad lib smoking) and a second sample four weeks after a target quit day. Additional information around the study protocol has been published elsewhere [157]. This study was approved by the Tasmania Health and Medical Human Research Ethics Committee.

5.4 Smokers' urine sample preparation

5.4.1 Free NNAL analysis

Smokers' urine samples were stored at -20 °C prior to analysis. Following thawing at ambient temperature, the samples were centrifuged at 2000 *g* for 5 min. For free NNAL analysis, aliquots of each urine sample (adjusted to pH 7 with ammonia solution, 2 mL) were spiked with 40 µL of NNAL-d_3 (5 ng/mL) equivalent to 100 pg/mL of urine and mixed. An equal volume of ethyl acetate (2 mL) was then added to the urine solution. The tube containing spiked urine and solvents was shaken for 1 min by hand and vortexed for 3 min to mix urine

and solvents. The solution was kept for 5 min at ambient temperature to allow the separation of the solvent phase and aqueous phase, and then all the samples were centrifuged at 2000 *g* for 8 min. The supernatant organic phase was transferred into a glass vial. The extraction process was duplicated to all the samples. The supernatant organic phase was evaporated to near dryness by using nitrogen gas. All the samples were then reconstituted with 100 µL of deionised water before using UPLC-MS/MS to determine NNAL levels.

5.4.2 Total NNAL analysis

The total level (free NNAL + glucuronide conjugate) of NNAL was determined by enzymatic hydrolysis of urine based on methods employed previously [124, 126, 155]. The enzyme solution was prepared by dissolving *β*-glucuronidase Type IX-A enzyme in phosphate buffer saline (pH 7.4, 10 mM phosphate buffer, 2.7 mM potassium chloride and 137 mM sodium chloride) equivalent to an enzyme activity of 40,000 units/mL. Aliquots of each urine sample (2 mL) were spiked with 40 µL of NNAL-d_3 (5 ng/mL) equivalent to 100 pg/mL of urine and mixed. The urine solutions were treated with 100 µL of *β*-glucuronidase enzyme solution equivalent to 2000 units/mL of urine. The samples were vortexed and placed in an incubator at 37 °C for 20 hours in the dark. The hydrolysed urine samples then used the same extraction process as free NNAL analysis. The calibration standard samples were processed using the same extraction procedure.

5.5 Results and discussion

5.5.1 Assay performance

The assay validation parameters including precision, accuracy, LoD and LLoQ met the acceptance criteria. A representative chromatogram of a smoker's urine sample with a level of free NNAL 45 pg/ml (at the lower range of NNAL samples) is shown in Figure 5.1. The

average recovery of free NNAL extracted from the smokers' urine samples was 59.6% ± 23.5 (mean ± SD).

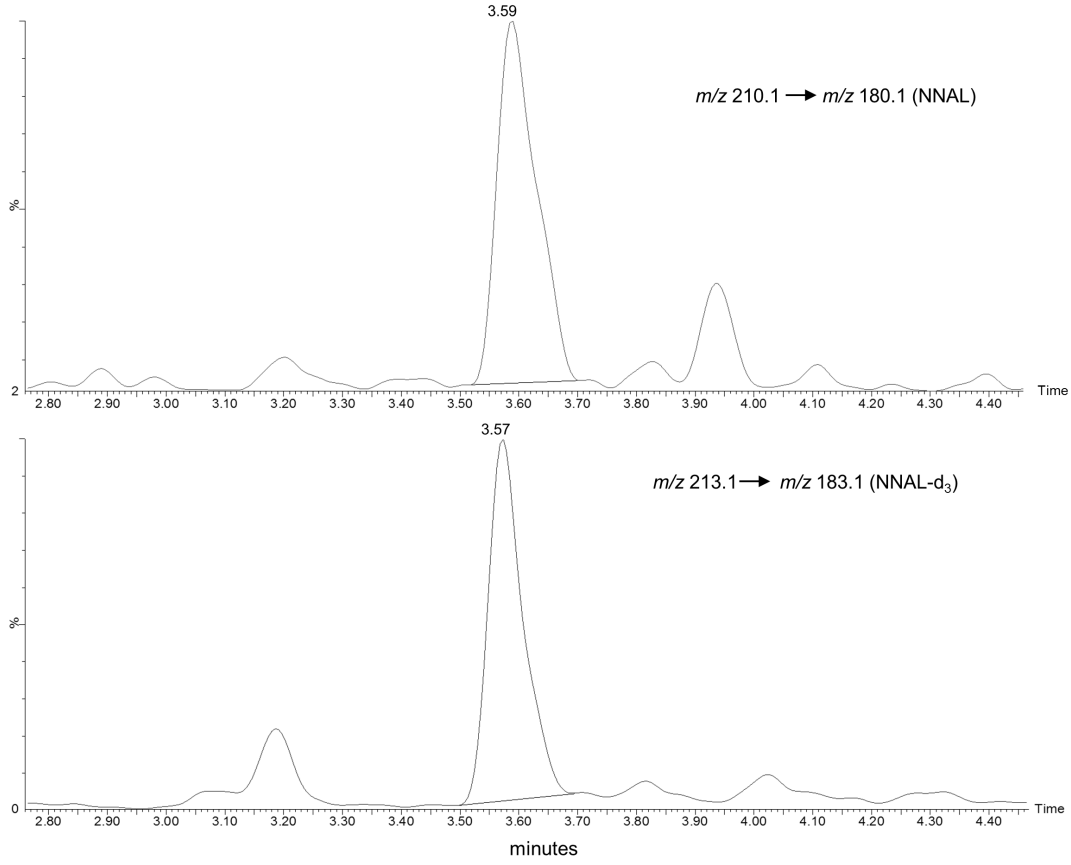

Figure 5.1 Representative UPLC-MS/MS chromatograms of free (unconjugated) NNAL and NNAL-d_3 from a smoker's urine sample with a concentration of NNAL of 45 pg/mL

5.5.2 NNAL levels in smokers' urine

The average free NNAL level in the smokers' samples (n= 24) prior to starting their quit attempt was 193.52 ± 158.17 pg/mL (mean ± SD); this fell to 64.49 ± 77.59 pg/mL four weeks after the quit attempt (p<.002; Figure 5.2). As expected, NNAL levels were significantly reduced following the quit attempt; this supports the use of NNAL as a biomarker of tobacco-related exposure and harm minimisation. Other studies have determined the total NNAL level (free + glucuronide) in urine samples [115, 126, 155].

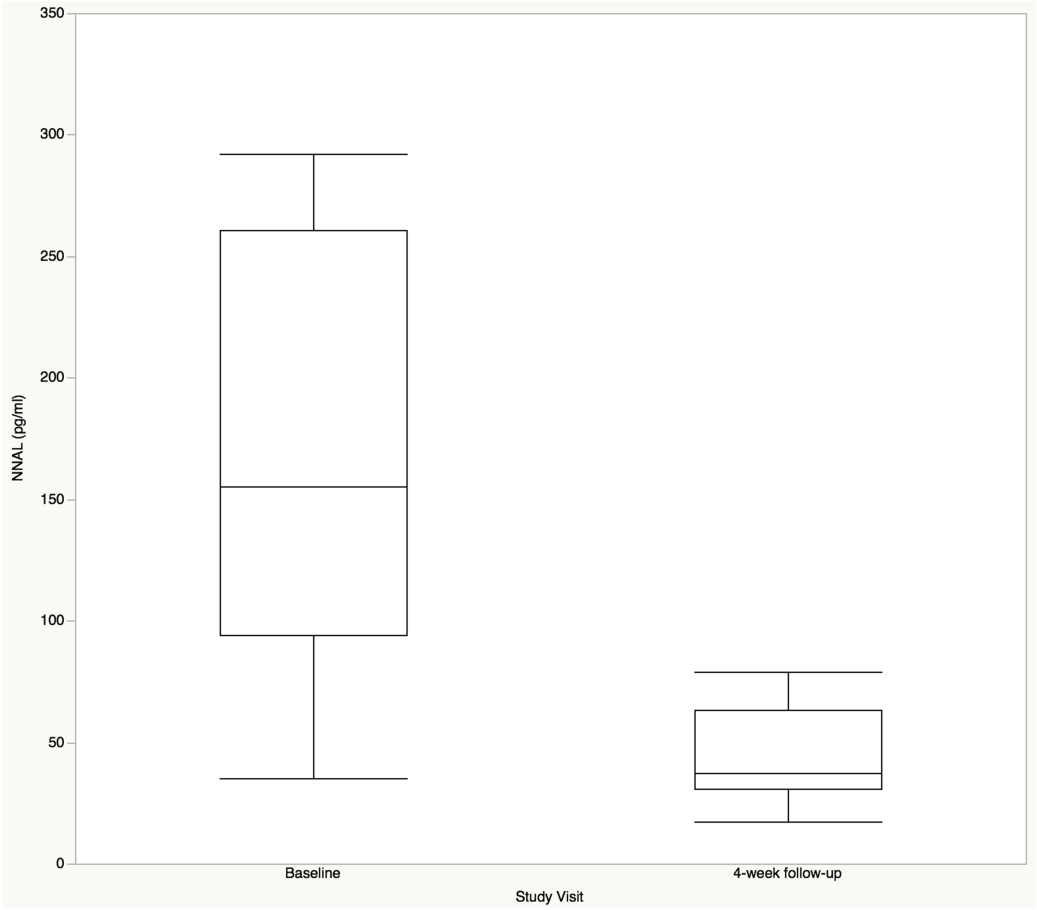

Figure 5.2 The box-whisker plot represents the range of NNAL levels measured from smokers' urine samples. The bottom and top of the box represent the first and third quartiles of NNAL levels; the band inside the box represents the median of NNAL levels; the ends of the whiskers represent the standard deviation above and below the mean of NNAL levels

Total NNAL levels were measured using the β-glucuronidase enzyme to hydrolyse NNAL-glucuronide conjugates to free NNAL aglycone. However, it was found that under previously reported standard hydrolysis conditions [124, 126, 155], the ratio of total NNAL to free NNAL was <=1 for some urine samples in our sample population (Figure 5.3). Enzymatic hydrolysis was clearly unreliable and there was a potential for significant loss of either free NNAL and NNAL aglycone. Figure 5.4 shows the loss of NNAL analyte from a smokers' urine sample following enzymatic hydrolysis. Possible mechanisms for this loss may be that the aglycone is unstable in the hydrolytic conditions of the standard procedure, or binds non-specifically to the β -glucuronidase or matrix [156]. Hence, based on our findings, indirect glucuronidase hydrolysis approach is not recommended. It is worth noting that three recent studies [124, 126, 155] have not adequately undertaken validation of measuring NNAL aglycone, but rather inferred validation using results from free NNAL analysis or repeat hydrolysis of the same samples. Proper validation of indirect methods such as β – glucuronidase hydrolysis to measure total NNAL (free + glucuronide) or conjugated NNAL by difference (as used in the detoxification ratio) should utilise NNAL-glucuronide standards.

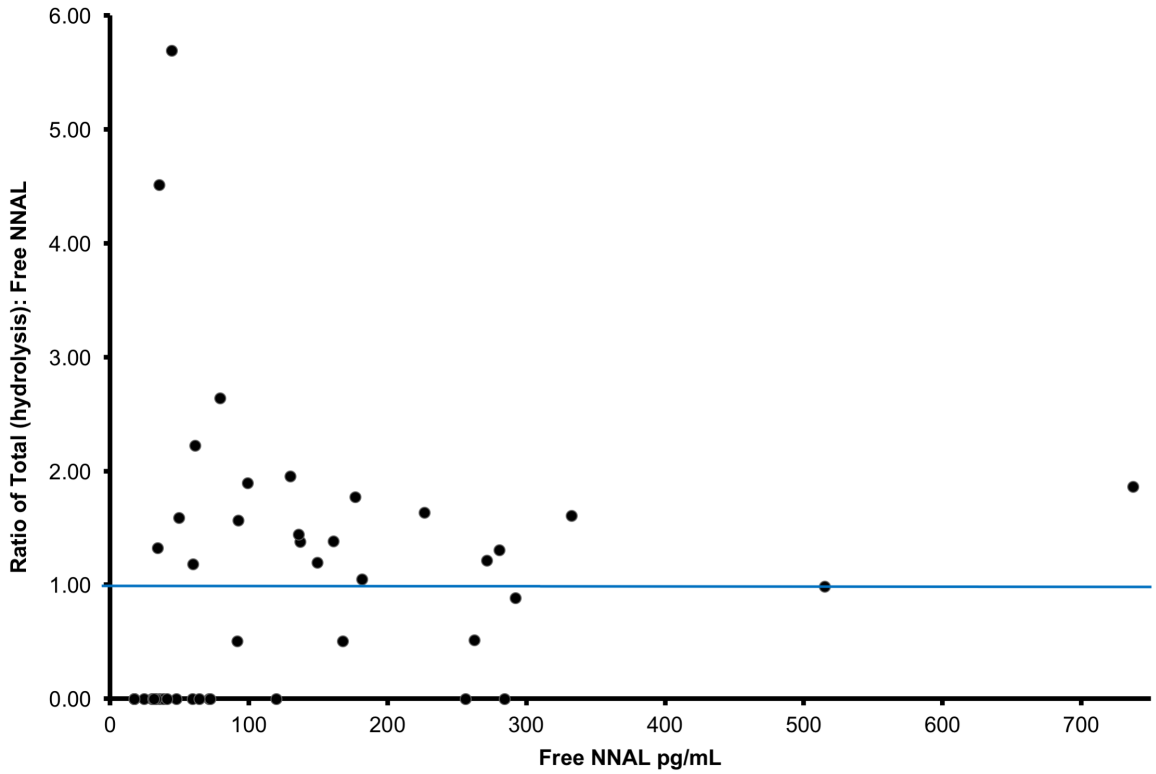

Figure 5.3 Ratio of total NNAL (aglycone measured after hydrolysis) to free NNAL, where a ratio <= 1 indicates loss of NNAL aglycone caused by the β-glucuronidase hydrolysis procedure

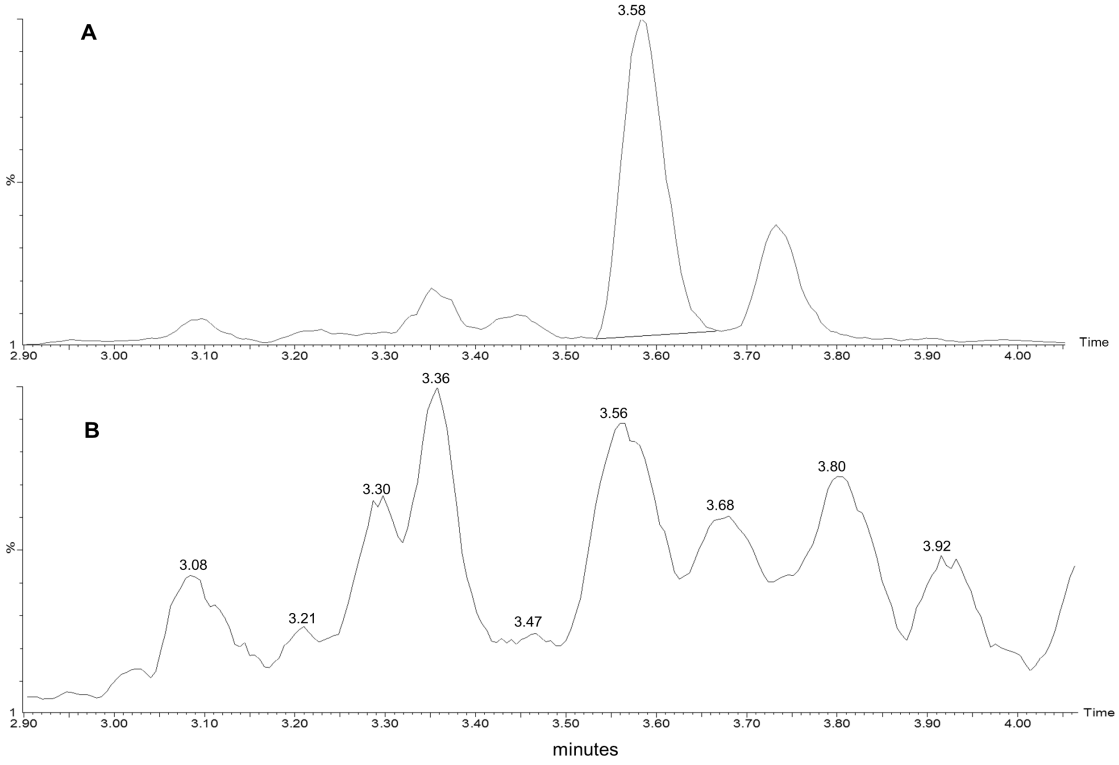

Figure 5.4 UPLC-MS/MS chromatogram of a representative urine sample before (A; free NNAL at 256 pg/mL) and after (B) β-glucuronidase hydrolysis under standard conditions, showing loss of NNAL aglycone to below method detection limit following hydrolysis

Our previous study demonstrated, to determine total levels of metabolites, indirect methods of measuring glucuronide metabolites using *β*-glucuronidase hydrolytic cleavage were unreliable for metabolites of nicotine [128]. Proper validation of indirect methods such as *β*-glucuronidase hydrolysis to measure total NNAL (free + glucuronide) or conjugated NNAL by difference (as used in the detoxification ratio) should utilise NNAL-glucuronide standards.

5.6 Conclusion

In summary, NNAL can be accurately quantified with a single ethyl acetate extraction. This new assay is an improvement over existing SPE-based methods in that it is substantially less complicated while maintaining adequate sensitivity. It is a straightforward approach to measuring the NNAL level in smokers' urine. Application of the assay was successfully used to assess tobacco-related harm exposure and the effectiveness of smoking reduction harm minimisation strategies. Enzymatic hydrolysis of NNAL-glucuronide as a technique for measuring the aglycone is not recommended.

Chapter 6: The Effect of Varenicline and Nicotine Patch on Smoking Rate and Satisfaction with Smoking: An examination of the mechanism of action of two pre-quit pharmacotherapies

Chapter 6

6.1 Background

Every year, millions of people die from tobacco smoking and suffer from tobacco-related diseases [1, 158]. After not commencing smoking in the first place, encouraging successful cessation is the most effective strategy for reducing smoking-related harm [9]. Currently, smokers can choose from a number of efficacious, if clearly imperfect, cessation medications and methods to aid a quit attempt [66]. Smoking cessation medications, chiefly nicotine replacement therapies and varenicline, are considered first line treatments [159, 160] for smokers wishing to quit.

Until relatively recently, smokers attempting to quit with nicotine replacement therapies were restricted to abrupt cessation attempts. That is, they were advised to stop smoking entirely prior to stating their quit attempt. More recently, some guidelines for smoking cessation (e.g., in the U.K.[70]) have made recommendations to allow smokers to use medications – typically nicotine replacement – to aid gradual reduction prior to a quit attempt. Reduce to quit approaches are underpinned by evidence that smoking reduction prior to quitting improves a smoker's odds of successful cessation [29, 31, 34, 47, 161]. There is also evidence that successful smoking reduction actually promotes interest in quitting [33], and a suggestion that it is a useful treatment for smokers who are currently disinterested in complete cessation [9].

Nicotine patch is the mostly widely used form of Nicotine Replacement Therapy (NRT) and was originally designed to be initiated on a patient's quit day [162, 163]. In recent years, however, nicotine patch has been used by starting treatment 1-2 weeks prior to quitting (known as preloading [74]), and followed by a standard post-quit patch regime. Our group has reported that pre-quit nicotine patch reduced the satisfaction with smoking and daily

cigarette smoked during the pre-quit period, and theorised that the reduced satisfaction might drive the smoking reduction and, in turn, cessation [133]. This finding is in line with earlier studies which reported that smokers rate smoking less satisfying while they were wearing nicotine patch [164, 165]. However, our study was a small (n= 61), single-group feasibility study and did not have a control group, rendering us unable to determine whether pre-quit patch treatment might be a causal factor in the reduction of satisfaction and daily smoking rate during the pre-quit period as opposed to these variables simply naturally dropping in the lead up to a quit attempt. To overcome this limitation, this current study was designed to include a control group of participants who obtained nicotine patch treatment on quit day. In addition, varenicline is the newest pharmacotherapy treatment and has been found more effective than a single type of NRT [55]. Varenicline has been reviewed in literature that it can increase the sustained abstinence rates in long-term smoking cessation [166]. The use of varenicline starts 1-2 weeks prior to quit day, similar to the pre-quit patch regime. The efficacy of varenicline might also be mediated via the reduction in satisfaction gained from smoking, but similarly to pre-quit patch treatment, this mechanism has not explicitly been tested before [57]. Understanding how these two pre-quit treatments impact the reduction of smoking, and whether the type of pre-quit treatment makes any difference on satisfaction with smoking during the pre-quit period may help to better tailor treatment for an individual.

The study was designed as a three-group, randomised, open-label study [157]. The primary objective of this study was to examine the effects of the pre-quit use of varenicline and nicotine patch on the daily smoking rate and satisfaction with smoking, and test the relationship between these variables during the two weeks leading up to a quit attempt. Participants were randomised to either a standard patch group that started patch treatment from an assigned target quit day, or one of two pre-quit treatments groups, which receive

either pre-quit patch or varenicline two weeks prior to a quit day, all followed by ten weeks of post-quit treatment. We hypothesised that 1) satisfaction with smoking would decline during the two week pre-quit period for smokers treated with patch or varenicline (compared to smokers in the standard patch group); 2) the smoking rate would decline during the two week pre-quit period for smokers treated with patch or varenicline (compared to smokers in the standard patch group); and 3) satisfaction with smoking would mediate the effect of pre-quit treatment on smoking reduction during the two week pre-quit period. Finally, we examined differences in smoking reduction and satisfaction among the treatment groups. We mainly focused on effectiveness of pre-quit treatment on reduction of smoking rate and satisfaction with smoking in Chapter 6. Adding biological measures will confuse the main purpose of this chapter. In addition, the biological measures were reported in Chapter 5, page 73. Therefore, the biological measures were not included in Chapter 6.

6.2 Methodology

6.2.1 Participants

Participants who were interested in quitting were recruited mainly through advertisements on social media [167, 168] and local newspapers. Screening established eligibility according to the inclusion criteria: Being 18 years of age or older; current daily smoking ≥ 10 cigarettes per day (CPD) for at least the last 3 years; having a high motivation to quit smoking (defined as scoring ≥ 75 on a 100 point motivation scale; [169]); and willing to use either patch or varenicline as part of a quit attempt. Participants were excluded if they were currently enrolled in a smoking cessation trial, or had been within the past three months or were unsuitable for treatment with either patches or varenicline. The study was approved by the Tasmanian Health and Medical Human Research Ethics Committee (H0013619).

6.2.2 Treatments

Participants were randomised to two pre-quit groups (nicotine patch or varenicline) or to a standard patch group without pre-quit treatment. Participants in the pre-quit groups were provided either nicotine patch (21mg/24 hr) or varenicline for two weeks prior to their target quit day. Participants in the standard patch group started patch treatment on their target quit day and did not receive any treatment during the pre-quit period.

All groups then received ten weeks of post-quit treatment. Participants in the two patch groups remained on the same dose (21mg/24 hr) for ten weeks following their assigned quit day; the dose was not titrated downwards over the course of the study [170]. With varenicline treatment, participants received 1 mg twice per day following a one-week titration period (0.5 mg once per day for three days, followed by 0.5 mg twice per day for four days). Participants were asked about the treatment use, including frequency of use and adverse events, at each study visit.

6.3 Procedures

6.3.1 Baseline visit.

The detailed study procedures can be accessed in our published study protocol (Appendix I) [157]. Briefly, participants completed five study visits over the course of the study: Enrolment visit (Day -17), Treatment assignment visit (Day -14), Quit day visit (Day 1), Follow-up visit (Day 14), Final visit (Day 28). At the Enrolment visit, participants were firstly given a detailed overview of the study requirements and asked to provide written informed consent. Participants were then randomised to the treatment group and assigned a target quit day. Participants were asked to complete baseline data collection during the Enrolment visit. This consisted of demographic (e.g., age, gender, ethnicity etc.), smoking

history characteristics (e.g., cigarettes per day [CPD], years smoked, interest in quitting etc.), and information about satisfaction with smoking (modified Cigarette Evaluation Questionnaire [mCEQ] [171]). Next, participants were asked to complete a 14-day timeline follow-back (TLFB) [172] and to provide two exhaled carbon monoxide (CO) samples. Both the TLFB questionnaire and CO measurements were repeated at all subsequent study visits.

6.3.2 Ecological Momentary Assessment (EMA) data collection

From enrolment until two weeks after their quit day (approximately 28-days of monitoring in total) participants were required to monitor their smoking behaviour, affect and activities in real-time conditions using a smartphone running study-specific software [173]. The EMA monitoring procedures were modelled on previous smoking studies [174, 175]. Participants were required to carry the device with them at all times and to indicate (by tapping a button) each time they smoked a cigarette; ~4-5 cigarettes per day were randomly selected for assessment during which participants were asked to report on their current affects, location and activities. Along with smoking assessment, participants completed ~4-5 randomly-timed non-smoking assessments ("random prompts), each day. These assessments largely paralleled those administered during smoking assessments but also included a question on satisfaction with the most recently smoked cigarette (rated on a 0-100 point scale). Participants were required to complete an evening assessment between 7 pm and midnight. During this assessment, participants reported on daily mood and satisfaction with smoking (rated on a 0-100 point scale), among other items (see [157] for further details). EMA data was downloaded and checked at each study visit with additional training provided when compliance with monitoring was less than 100% [174].

6.4 Data cleaning and analytic plan

To explore the study hypotheses, we used CPD based on TLFB reports collected at study visits; daily satisfaction with smoking was taken from the evening reports where available, and from the mean of that day's random assessments if random prompt responses were available when an evening report was not. We also analysed the data using real-time CPD counts and found similar results (data not presented).

The effects of pre-quit treatment on both satisfaction with smoking and the number of CPD were assessed using linear mixed models to account for the multi-level structure of the data (repeated measures on individuals over time). The models were fitted using Stata statistical software (Stata 12.1). Random intercepts were incorporated into the models to represent individual differences in CPD and satisfaction at the start of the pre-quit period. An interaction of treatment group by time was included in the models to assess the differences in mean outcome trajectories in the three treatment groups over time. Random intercept models incorporating squared and cubic trends for time and their respective group by time interactions were compared with the simpler random intercept models with a linear trend for time and a group by time interaction. In both cases the linear time trend was a better fit. The fit of a random slopes model was then evaluated. For both CPD and satisfaction, a model including random slopes was found to be a better fit than the simple random intercepts model. The final models thus incorporated both random intercepts and random slopes, a linear trend for time and an interaction of time by the treatment group.

To assess the extent to which between-person difference in satisfaction mediated the effect of treatment on cigarettes smoked per day, the effect of treatment on CPD was modelled with and without adjustment for satisfaction. This allowed us firstly to assess both

the direct effect of treatment on CPD, controlling for satisfaction, and the total effect of treatment on CPD, including the effect of satisfaction. This is a so-called lower level or 2→1→1 mediation [176], where the effect of a level 2 predictor (treatment group) on a level 1 outcome (smoking rate) is mediated by a level 1 predictor (satisfaction). Random intercepts and random slopes for both time and satisfaction were fitted to allow both change in CPD over time and change in CPD with change in satisfaction to vary across individuals. All covariates were grand-mean centred. As a final step, for each treatment group, the mean daily change in satisfaction that was associated with treatment was multiplied by the mean effect of a one-unit change in satisfaction on CPD, in order to determine the mean indirect effect of treatment on CPD, via satisfaction. To ascertain whether these indirect effects were significantly greater than zero, it was necessary to estimate the standard errors of the products of the two regression coefficients. Hayes and Preacher [177] suggest bootstrapping as the most reliable method of estimating variance in these parameters. However, the autoregressive specification for the residual correlation used in the above linear mixed models created problems with bootstrapping, as replacement of observations for bootstrapping resulted in repeated time measures within individuals. This model is unable to run with tied time measures for individuals. Another resampling method, the jackknife method, was therefore used in this analysis to estimate the standard errors: we ran the above linear mixed models multiple times with one subject omitted each time, and estimated the product of coefficients multiple times. The mean and standard deviation (SD) of the set of estimated products were then used to calculate the 95% confidence intervals (CI).

6.5 Results

6.5.1 Demographic of participants and data reduction

A total of 213 participants were enrolled and randomised to a treatment group (Table 6.1). The average participant was female (58%), Caucasian (93%), 42 years old, and smoked 19 CPD. The three treatment groups were evenly balanced, not differing on any of the characteristics measured (Table 6.1).

A total number of 213 participants were enrolled, but n= 30 withdrew from the study without providing either TLFB or EMA data during the pre-quit period. Thus, there were 183 participants who were eligible for analysis: 60 eligible participants in the standard patch group, 62 in the pre-quit patch treatment group and 61 in the varenicline group. The resulting dataset comprised 2,562 potential participant-days of observation. Of the 183 participants, 95 (51.9%) recorded at least some of the data on all 14 pre-quit days, and 153 (83.6%) recorded data on at least 7 days. The mean number of days with data recorded was *M*= 11.39 days per participant [standard deviation (*SD*)= 3.98].

Table 6.1 Demographic and smoking characteristics: overall, and by treatment group

	Treatment group			
	Standard patch (n= 69)	**Pre-quit patch (n= 72)**	**Varenicline (n= 72)**	**Total (n= 213)**
Age (mean, SD)	39.7 (11.1)	43.8 (13.4)	43.1 (12.2)	42.2 (12.3)
Gender: % female (n)	50.7 (35)	63.9 (46)	59.7 (43)	58.2 (124)
Race: % Caucasian (n)	91.3 (63)	93.1 (67)	93.1 (67)	92.5 (197)
Post-year 12 education: % (n)	57.4 (39)	45.1 (32)	52.8 (38)	51.7 (109)
Employed: % (n)	72.5 (50)	56.9 (41)	80.6 (58)	70.0 (149)
Cigarettes per day (mean, SD)	17.9 (7.0)	19.4 (9.8)	19.3 (7.0)	18.8 (8.0)
Time to first cigarette ≤ 30 min: %(n)	69.6 (48)	76.4 (55)	76.4 (55)	74.2 (158)

6.5.2 Reduction in CPD and satisfaction during pre-quit period

There was a significant linear reduction of 0.34 cigarettes per day over the pre-quit period in the pre-quit patch group (95% CI= 0.23-0.45) and 0.39 cigarettes per day in the varenicline group (95% CI= 0.28-0.51) (Table 6.2; Figure 6.1). The difference in the daily reduction in CPD between the pre-quit patch and varenicline groups was not significant (p= 0.49). CPD did not change significantly over the pre-quit period in the standard patch group (mean daily reduction in CPD= 0.05, 95% CI= -0.07-0.17).

Mean satisfaction with smoking decreased by 0.8 points per day in the pre-quit patch group (95% CI= 0.32-1.29) and 1.8 points per day the varenicline group (95% CI= 1.31-2.28) (Table 6.3, Figure 6.2). Change in satisfaction per day was significantly greater in the varenicline group than the pre-quit patch group (p= 0.005). Satisfaction with smoking did not change over the pre-quit period in the standard patch group.

Table 6.2 Daily reduction in mean cigarettes per day (CPD) for each treatment group over the pre-quit period: Results from linear mixed models

Treatment group	Daily reduction in CPD (β)	Standard error (SE)	95% Confidence Interval	p1 *	p2 **	p3***
Standard patch	0.05	0.06	-0.07, 0.17	0.45	-	-
Pre-quit patch	0.34	0.06	0.23, 0.45	<0.001	<0.001	-
Varenicline	0.39	0.06	0.28, 0.51	<0.001	<0.001	0.49

*p1= p-value from Wald test of interaction coefficient estimating change in cpd over time (versus null hypothesis of no change).
**p2= p-value from test of difference in change over time in respective treatment group versus standard patch.
***p3= p-value from test of difference in change over time in pre-quit patch group versus Varenicline group.

Table 6.3 Daily reduction in mean satisfaction with smoking for each treatment group over the pre-quit period: Results from linear mixed models

Treatment group	Daily reduction in satisfaction (β)	Standard error (SE)	95% Confidence Interval	p1*	p2**	p3***
Standard patch	-0.08	0.24	-0.56, 0.40	0.73	-	-
Pre-quit patch	0.80	0.25	0.32, 1.29	0.001	0.01	-
Varenicline	1.80	0.25	1.31, 2.28	<0.001	<0.001	0.005

*p1= p-value from Wald test of interaction coefficient estimating change in satisfaction over time (null hypothesis= no change over time).
**p2= p-value from test of difference in change over time in respective treatment group versus standard patch (null hypothesis= no difference).
***p3= p-value from test of difference in change over time in pre-quit patch group versus varenicline group (null hypothesis= no difference).

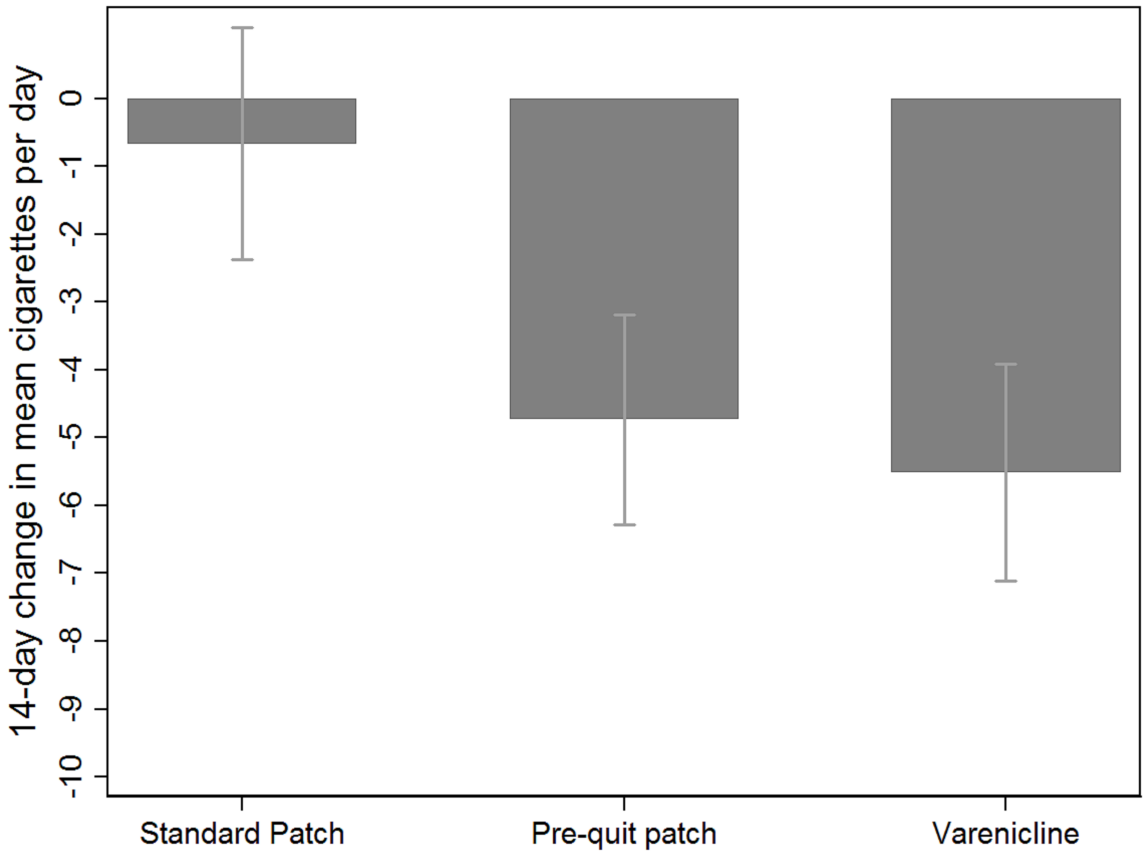

Figure 6.1 Mean (and 95% confidence intervals) reduction in cigarettes per day (CPD) over the pre-quit period in each of the three treatment groups

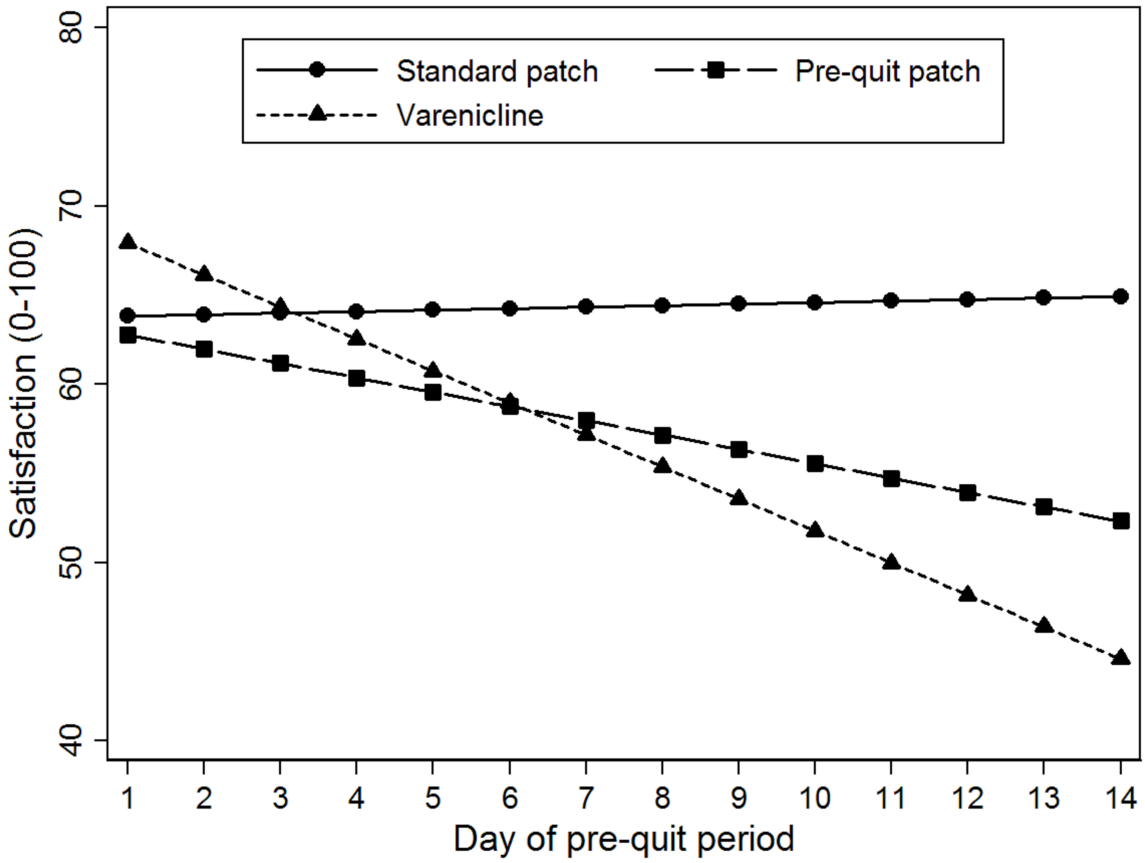

Figure 6.2 Modelled reduction of satisfaction with smoking over the pre-quit period in each of the three treatment groups

6.5.3 Relationship between treatment, satisfaction and CPD

In the mediation analyses, we were interested in investigating the extent to which the association of effect of treatment on satisfaction was mediating the effect of treatment on CPD. Figure 6.3 shows the mediation model. The standard patch group experienced no significant daily reduction in satisfaction in smoking, and no significant reduction in cigarettes per day across the pre-quit period. In both the active treatment groups there was a significant direct effect of treatment on CPD, as shown by the c'_1 and c'_2 pathways. In the pre-quit patch group the direct effect was a mean daily reduction of 0.29 cigarettes per day

(95% CI= 0.19-0.39). In the varenicline group the direct effect was a mean daily reduction of 0.32 cigarettes per day (95% CI= 0.22-0.43). Both active treatment groups also experienced a daily reduction in satisfaction with smoking, which had an additional small but significant effect on cigarettes smoked per day, calculated as a_1*b and a_2*b (an additional reduction in cigarettes per day of 0.014 (95% CI= 0.012-0.015) and 0.035 (95% CI= 0.033- 0.037) in the pre-quit patch and varenicline groups, respectively). The total daily reduction in cigarettes per day associated with treatment is $c_i = c'_i + a_i$*b for each group i= 0 to 2. This is 0.30 CPD in the pre-quit patch group and 0.36 CPD in the varenicline group. The percentage of the change in CPD mediated by satisfaction is 4.6% in the pre-quit patch group and 9.9% in the varenicline group. Note that the total daily reductions in satisfaction and CPD differ slightly from those described above, as the mediation analysis was restricted to subject-days for which satisfaction with smoking was recorded (76% of all subject-days).

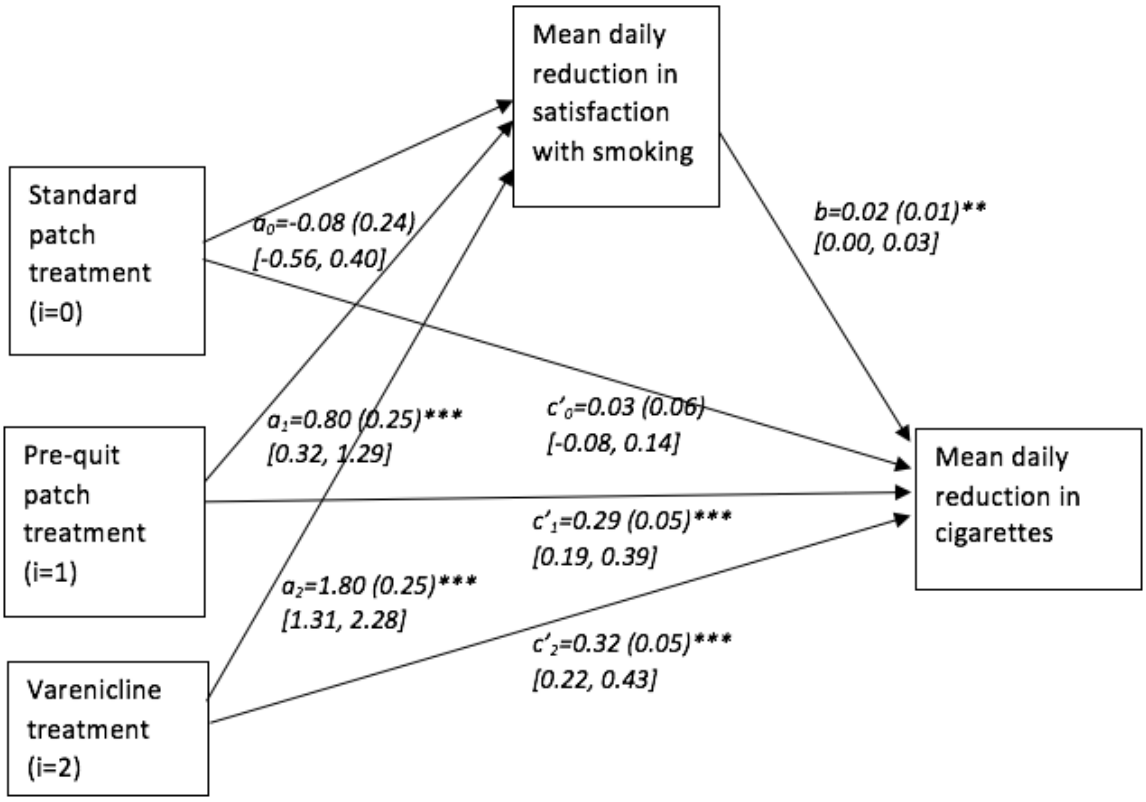

Figure 6.3 Mediation Model. Notes: Each of the three treatments has direct effects on satisfaction and daily smoking rate. Satisfaction with smoking meditates daily smoking rate. All coefficients with subscripts are the associations of the relevant treatment with the outcome (in direction of arrow). Standard errors are in parentheses, 95% confidence intervals in square brackets. **p< 0.01; ***p< 0.001

6.6 Discussion

This study was designed to test the effect of pre-quit treatment – either with nicotine patch or varenicline – on smoking reduction and satisfaction with smoking. The results will help elucidate the potential mechanisms driving the reduction during the pre-quit period. Compared to standard patch, as hypothesised, there was 1) a significant reduction in satisfaction with smoking during the pre-quit period with both pre-quit treatments; and, 2) a significant reduction in CPD during the pre-quit period with both pre-quit treatments. These

results paralleled with our previous finding [133]. Furthermore, the results from the mediation analyses were consistent with the notion that the pre-quit treatments caused a reduction in satisfaction with smoking during the pre-quit period, which in turn resulted in fewer cigarettes smoked. Treatment with varenicline resulted in a greater decrease in satisfaction with smoking compared to pre-quit patch. This finding consistent with previous reported results that varenicline treatment results in greater decrease in the satisfaction than nicotine patches [178]. Varenicline binds with the nicotine acetylcholine (Ach) receptor, acts as a partial antagonist to prevent inhaled nicotine from activating the Ach receptor sufficiently to cause the pleasure and reward response [179]. In addition, the pre-quit treatments did cause a reduction effect on CPD during the pre-quit period, although the difference in the daily reduction in CPD between pre-quit patch and varenicline was not significant. Previous research has demonstrated that the pre-quit nicotine patch [180] and varenicline [166] reduced the satisfaction and cigarette consumption, as well as improved the abstinence rates.

Our finding indicated that the reduction of satisfaction with smoking was observed with two pre-quit treatments during pre-quit period; and the declined satisfaction was associated with the reduction in daily smoking rate ($p< 0.01$). The results from the mediation analysis were as hypothesised: a relatively small, but significant, proportion of the total reduction in CPD could be attributed to the reduction of satisfaction with smoking in both pre-quit patch and varenicline groups. Satisfaction with smoking was hypothesised to mediate the effect of treatment on smoking reduction; as such, satisfaction with smoking might be a plausible marker of treatment response, potentially allowing for better tailoring of mediating mechanisms for the pre-quit treatment in smoking reduction, pre-quit treatment duration, or both. However, the relatively weak relationship between satisfaction and smoking reduction

suggest that satisfaction may not be a viable method of evaluation responsiveness to treatment. One previous study reported that varenicline promoted reduction in smoking rate, dependence and craving [57]. However, it was not further explained whether the reduction of smoking rate was mediated via reducing craving or dependence. Examining the relationship between these variable – and others – and smoking reduction may prove fruitful in the search for useful markers of treatment response.

All participants received open-labelled treatments, so the observed reduction in satisfaction and smoking rate may be influenced by other factors than treatment. However, previous studies have demonstrated both pre-quit nicotine patch [133] and varenicline [57] caused reduction in satisfaction with smoking and smoking rate.

6.7 Implications and conclusions

The results suggested that pre-quit treatment use caused reductions in satisfaction with smoking and smoking rate. Satisfaction was associated with the reduction in smoking rate, although the mediation effect of satisfaction was weak. There might be other variables, such as craving and smoking dependence, with pre-quit treatment use that can be used to mediate smoking reduction. Our previous study has found there was reduction on craving with pre-quit patch use in low smoking dependence smokers over the course of the pre-quit period [133]. Monitoring such reductions may prove a useful method of evaluating responsiveness to treatment and allow for tailoring of treatment. For example, future studies may want to test whether reduced satisfaction leads to reduced craving and dependence, which in turn predicts reductions in smoking rate.

Chapter 7: General Discussion

Chapter 7

Tobacco harm reduction strategies have made great advances in helping those who smoke to reduce the harm related to smoking. Smoking cessation has played an important role in the tobacco harm reduction. A variety of interventions have been applied to assist the achievement of cessation, however, the treatment outcomes have not been as efficient as expected. Studies have suggested that smokers were unable to obtain enough nicotine with existing regulator-approved NRT products, or struggled to quit completely, or found it difficult to sustain abstinence for a long period. Alternatively, smoking reduction with treatment has helped smokers to reduce smoking, either as an end itself or a step toward complete abstinence. Determination of how the use of treatment affects smoking reduction prior to complete abstinence has been useful, along with results on what the consequences of smoking reduction has been on the smoker. The objective of this thesis was to develop enhanced methods of examining harm exposure, specifically the development of a nicotine-related biological marker in urine, and an assay to quantify the components of VNPs. Additionally, I planned to explore the drivers of reduction in the context of a quit attempt.

A range of interventions used to assist smoking reduction. The new form of nicotine delivery products – VNPs – have caught the attention of smokers and these have become more and more popular in recent years. They have been considered as a tool, for reducing or quitting smoking, even though there were concerns on safety and unclear efficacy on cessation. Due to lack of product regulation, unlike NRT, the consistency of nicotine content in e-cigarettes was not clearly known. There are a variety of types of e-cigarette cartridges, such as fibrous material pad based cartridges and liquid cartridges. Because of the uncertain amount of nicotine and the varied form of cartridges, it may make the determination of the effectiveness of VNPs use in smoking reduction. A few assays have been designed to analyse the nicotine content in e-cigarette cartridges. However, these assays have been applied to

liquid cartridges, or refill solutions. A few assays have analysed the nicotine content in fibrous pad based cartridges, however, the internal standards used in those assays had different chemical properties from nicotine. Therefore, the nicotine content in these cartridges may not be accurately represented. An efficient and accurate assay was developed and applied to determine the nicotine content in e-cigarettes, which consisted of a fibrous pad in the cartridge (Study 1). As results in our laboratory and others have shown that even nicotine free labelled cartridges may contain nicotine [105]. It can be a potential concern if given these VNPs as a control feature in a smoking study. This assay has been particularly useful where blank cartridges are not available. In this assay, a known content of nicotine-d_4 was used as a standard spiked onto the fibrous cartridge pad material, and then compared with the nicotine-d_4 standard, using external standardisation to calculate the recovery of nicotine-d_4 from the pad. This overcame the limitation of previous studies that had minimal validation with respect to recovery of analyte from the cartridge pad [105, 131].

With this well-established methodology, assay performance validation criteria, including accuracy and precision, LoD and LLoQ, freeze/thaw and bench top stability, were validated. The indication from this assay shows that there was good performance for determining the nicotine content in e-cigarette cartridges. In addition, the assay was applied to one brand of e-cigarettes, to determine the nicotine content and compare the variation between batches and within batches. This assay also showed that nicotine content did not match what was claimed on the label. Significant differences (~25%) were found between cartridges labelled and the actual content of nicotine in e-cigarette cartridges. This finding indicated that the information regarding the nicotine content found on the product package provided by manufacturers, may mislead customers and therefore the direct comparison with e-cigarettes is difficult. Knowing the nicotine content in e-cigarettes is of particular

importance when interpreting the outcomes of studies designed to evaluate the safety of e-cigarettes and / or their usefulness for smoking cessation and / or smoking reduction; particularly given the rate at which new products are emerging. Future studies examining the effectiveness of VNPs as either smoking reduction or smoking cessation aids will be able to use our assay to accurately report the nicotine content of the products used. This assay also will be able to aid researchers and policy makers who try to compare results across studies using different devices.

Apart from examining the treatment product itself, biological markers can be used to indicate the intake of nicotine and the exposure of smoking-related harm. The metabolites – NNAL and its glucuronides – are considered important biomarkers for NNK intake. Glucuronidation is an important NNK detoxification pathway. As discussed earlier, two isomers of NNAL-glucuronides, NNAL-N- and NNAL-O-glucuronide, are formed during the glucuronidation process. It is interesting to know whether the isomers of NNAL-glucuronides have potential importance to represent the NNK. Previous study indicated that liver microsomes from smokers (n=14) exhibited no significant difference in the levels of either NNAL-N-glucuronide or NNAL-O-glucuronide formation (not enantioselective), or in the ratio of NNAL-N-glucuronide/NNAL-O-glucuronide formation, as compared with liver microsomes from never smokers (n = 28) [181]. In addition, previous research has shown that (S)-NNAL and its glucuronides predominated in urine, with (S):(R)-ratios increased seven days after cessation. This indicated (S)-NNAL was retained in the body longer than (R)-NNAL. (S)-NNAL is considered the major enantiomer involved in the slow elimination of NNAL [182]. In addition, (R)-NNAL-O-glucuronide is inactive as a lung tumorigenic in A/J mice contrasted to the potent lung tumorigenicity of (S)-NNAL and NNK [183]. Hence, for future research of NNK intake from tobacco, there is a strong rationale to measure (S)-NNAL

and (S)-NNAL-N-glucuronide in smokers' urine. NNAL-N-glucuronides have been reported to contribute substantially to total combined NNAL-glucuronides. Therefore, free NNAL and NNAL-N-glucuronide are used as biomarkers of NNK intake from tobacco. To measure the levels of NNAL in smokers' urine, SPE assay was employed to reduce the interference that had been found from urine matrix in previously reported studies [124, 141]. However, the sample preparation procedures were complicated and difficult to replicate. Hence, a simple and efficient assay was developed (Study 2) to measure NNAL levels in smokers' urine while undergoing smoking reduction treatment.

As mentioned above (in Chapter 4), the reported SPE methods have been complex, with multiple preparation steps, which involved different stationary phases and solvents. To replace SPE, in this thesis, new types of liquid-liquid extraction methods, such as DLLME and DLLME-SFO, were considered when applied for NNAL extraction. These new extraction methods have advantages compared with the traditional liquid-liquid extraction assay, including minimal extraction solvent consumption, short extraction time. Therefore, both DLLME and DLLME-SFO and along with an additional three assays (derivatisation of NNAL, SPE and liquid-liquid extraction assay) associated with UPLC-MS/MS were developed for determining NNAL levels in urine samples. Efficacy of these assays was verified with the recovery of NNAL from urine samples. The recovery of NNAL was calculated based on the NNAL-d_3 concentration in urine samples and compared to a NNAL-d_3 standard, prepared to a concentration equivalent to recovery from the blank urine samples.

With the DLLME, the extraction solvent is required to have a lower solubility but a higher density than water, and the disperser solvent was used to assist the dispersion of the extraction solvent into the aqueous phase. However, the low NNAL recovery, that was not

efficient, especially given the low biological levels, limited the application of this assay for NNAL extraction. Similarly, the DLLME-SFO assay was not suitable for NNAL extraction. With the DLLME-SFO assay, 1-undecanol was used as the extraction solvent. 1-Undecanol has a low melting point (11 °C), which solidifies at ambient temperature (20 to 25 °C). 1-Undecanol also has less density than water, which would cause it to float above the aqueous phase, thereby making it easier for transferring the extract. However, the challenge with using 1-undecanol was it became too difficult to evaporate it directly with nitrogen gas, as 1-undecanol is highly lipophilic with a boiling point of 243°C. Even using other lipophilic and hydrophilic solvents to assist the evaporation process, 1-undecanol still could not be evaporated. Increasing the evaporation temperature (25, 32, and 45 °C) and the evaporation duration (15, 25 and 40 min) may have the potential to lose the analytes, either through chemical decomposition or evaporative loss. In addition, the UPLC column used in this study was narrower than the column used in previously reported studies. Thus, the UPLC column condition was not suitable for direct injection of 1-undecanol.

SPE assay with simplified extraction procedures was developed. One extraction solvent (acetonitrile) and one type of SPE cartridge were used to extract NNAL from urine samples. However, the NNAL chromatogram signals were embedded with background signals due to the matrix interference from urine. Thus, this simplified SPE assay was also found to be not suitable for NNAL extraction in urine samples.

As these three extraction assays were a failure for NNAL extraction, a liquid-liquid extraction assay was developed. The advantage of this assay is that NNAL can be accurately quantified with a single ethyl acetate extraction. The recovery of NNAL from urine samples (~65%) was greater than other assays (~25%) with this assay. The assay performance data

presented good accuracy results and precision values. Assay performance validated in blank urine met the bioanalytical quality control criteria [150]. The chromatogram also clearly presented a level of NNAL 45 pg/mL (at the lower range of NNAL samples) from a smoker's urine sample. Hence, liquid-liquid extraction assay is a good option for NNAL analysis and does not require extensive solid phase extraction sample preparation. This simple and efficacy liquid-liquid assay can be practically applied to evaluate the reduction of smoking-related harm exposure in future smoking study when use NNAL as a biological marker to determine harm exposure in smoking studies. Importantly, such biological markers are more accurate measures of exposure than self-reported smoking rates (as smokers can vary the "intensity" with which they smoke individual cigarettes, and hence to some extent compensate for a reduced number of CPD).

The validated liquid-liquid extraction assay was applied to smokers' urine samples, which had been collected from current smokers who were participants in a larger smoking cessation study (Study 3). The results of free NNAL levels in smokers' urine clearly indicated that there was a significant reduction following attempts by smokers to quit. This supports the use of NNAL as a biomarker of smoking-related exposure and harm reduction. In future smoking reduction studies, the impact of treatments on smoking reduction can be effectively evaluated by monitoring smoking behaviour, as well as by using biological markers – NNAL – to determine the actual harm exposure. Such designs will allow for a more accurate evaluation of the effectiveness of treatments in promoting smoking reduction.

Furthermore, this liquid-liquid extraction assay provided the evidence of the limitation of enzymatic hydrolysis of NNAL-glucuronide for measuring the NNAL aglycone, which is not recommended. The ratio of NNAL-glucuronide to total NNAL was proposed as

a potential biomarker [153] with rapid metabolism to the glucuronide inferring faster detoxification [154]. However, it was unclear what the ratio required would be with different findings between studies and ethnic groups [124, 126, 154, 155]. These reported methods were based on enzymatic cleavage of the NNAL-glucuronide to yield the aglycone and measure total NNAL after hydrolysis; this approach can lead to loss of accuracy and precision [156]. As we have shown, the NNAL-glucuronide measurement procedure (enzymatic hydrolysis approach to determine total levels) appears to be a major reason for the variability in the ratio of NNAL (free) to NNAL-glucuronide between individuals, but not the only reason. Variability could also be due to pharmacogenetic based differences in metabolism between individuals, differences in time between total cigarettes and last cigarette smoked, and differences in sample collection time relative to smoking pattern. Even for the glucuronides themselves, there is variability in NNAL-O-glucuronide and NNAL-N-glucuronide formation between individuals, and hence different pharmacokinetic profiles. The determination of total (free + conjugated) NNAL requires a proper analytical assay validation. In this study, total (free + glucuronide) NNAL levels were measured with the similar hydrolysis approach by using *β*-glucuronidase enzyme to hydrolyse NNAL-glucuronide conjugates to free NNAL aglycone. The results indicated that the ratio of total NNAL to free NNAL was below 1 for some urine samples in this sample population. And there was loss of NNAL aglycone from smokers' urine samples following enzymatic hydrolysis. The loss of NNAL aglycone might be due to aglycone becoming unstable in hydrolytic conditions in the standard procedure, or because it binds non-specifically to the *β*-glucuronidase or matrix [156]. NNAL-glucuronide measurement undertaken with standard enzymatic hydrolysis procedures is not reliable. Direct determination with using glucuronide standards would be highly recommended for future work. Our previous glucuronide study has indicated the direct determination could accurately measure the glucuronide levels in urine

samples [128]. The reason for not performing the direct analysis of NNAL-glucuronide was limited research time and costs in excess of the research budget. As discussed earlier, (S)-NNAL and (S)-NNAL-N-glucuronide are the major enantiomers in smokers' urine. The analytical assay (e.g. UPCL-MS/MS) can be applied to measure NNAL levels, specific to (S)-NNAL. In addition, the glucuronide standard approach to directly measure the NNAL-glucuronide levels in human urine samples should be used to perform a proper validation of indirect methods, such as β-glucuronidase hydrolysis to measure total NNAL (free + glucuronide) or conjugated NNAL by difference (as used in the detoxification ratio). A very recent research study has used NNAL-N-glucuronide standards with using LC/MS method to directly determine the levels of NNAL-glucuronides in urine samples [184]. This study successfully measured different types of NNAL-glucuronide isomers, including NNAL-N-glucuronides, (R)-NNAL-O-glucuronides and (S)-NNAL-glucuronide, in smokers' urine samples. The study indicated the association of UDP enzyme genotypes and the capacity of detoxification against NNAL, which may influence the cancer risk upon exposure to tobacco [184]. Utilisation of the biomarker assay to assess the urine samples of smokers is a potentially beneficial future strategy for smoking harm reduction. NNAL is a powerful carcinogenic biomarker from tobacco exposure. It is important to understand and monitor smoking reduction approaches with regard to exposures to tobacco-related harm, long-term follow-up of smoking reduction and / or cessation as well as to assess the risk of lung and other cancers.

Apart from determining the measures of therapeutic product itself and biological markers of harm exposure, it is also essential to understand the mechanism of the treatments promoted smoking reduction and allow to tailor the treatment consequences. A clinical trial involving two treatments – nicotine patches and varenicline – used two weeks prior to a quit

attempt, was conducted to explore the effect of treatments on smoking reduction and satisfaction with smoking, and the mediation relationship between smoking rate and satisfaction with smoking (Study 4). The study results indicated that both pre-quit treatments promoted a significant reduction in both the satisfaction with smoking and daily smoking rate from baseline to the end of the pre-quit period. This reduction was not observed in participants with standard patch regimen. The observed reduction of daily smoking rate could be attributed to the reduction of satisfaction with smoking, although the mediation effect of satisfaction was relatively small. Satisfaction with smoking might be a plausible marker of treatment response, potentially allowing for better tailoring of mediation type, pre-quit treatment duration, or both. However, the relatively weak relationship between satisfaction and smoking reduction suggest that satisfaction may not be a viable method of evaluation responsiveness to treatment. There would be other variables of reduction with pre-quit treatments, such as craving and smoking dependency. Furthermore, we used the NNAL assay that we developed in Chapter 4 to determine the tobacco-related harm reduction observed in Study 4. This data was presented in Chapter 5. The biological data was not included in the Study 4 (Chapter 6) because we focused on presenting the effectiveness of pre-quit treatment on reduction of smoking rate and satisfaction with smoking. For future studies, examining the relationship between cravings, dependence on smoking, and measuring the levels of biological markers may prove to be useful in evaluating responsiveness to treatment, risk of harm, and allow for tailoring of treatment.

Chapter 8: Conclusion

Chapter 8

Tobacco harm reduction aims to help reduce the morbidity and mortality of smoking related harm. Smoking cessation has been recommended as an effective strategy for tobacco harm reduction. More recently, smoking reduction is considered an alternate approach to reducing smoking harm exposure and may promote complete cessation. The reduction is quantified by measuring the constituents in nicotine products and measuring bioanalytical markers in biological fluid. The research purpose is to 1) develop improved measures of the constituents of medical nicotine products, particularly in VNPs; 2) develop measures of biological markers and apply these to assess smoking reduction; and 3) effectively use pre-quit treatments and accurately study smoking behaviour and its consequences. This thesis reported the development of assays for determining the nicotine content in new nicotine delivery products, as well as the development of an assay for measuring NNAL in smokers' urine, all measures that assist exploration of the drive for smoking reduction with nicotine treatments.

A variety of nicotine substitute treatments were used to assess the reduction, including new forms of nicotine delivery devices, known as VNPs. VNPs have increased the interest in helping to reduce cigarette smoking. To better measure more accurately the reduction for smokers while using VNPs, the constituents of VNPs must be clearly known before they can be used to measure the intake and exposure of smoking harm. In this thesis, an effective and accurate assay was developed to measure the nicotine content in the VNPs cartridges. This assay was important to determine the nicotine content in fibrous material pad based cartridges, especially as most existing assays have been designed to analyse nicotine content in liquid cartridges or refill solutions. In addition, this assay used deuterated nicotine, as an external standard, to quantify the nicotine content. This overcame the limitation of previous assays which included a standard with a different chemical property to nicotine. This analysis assay was successfully applied to determine the nicotine content in one brand of VNPs. The results

indicated a variation of actual nicotine content in the cartridge and to the content stated on the package label. VNP based products are currently unregulated and their market is rapidly evolving. These products vary in the types of delivery mechanisms used, and development of gold standard chromatographic methods to measure nicotine content need to be tailored to the particular device. VNP products have been developing rapidly, and methods to measure nicotine content have not kept pace, largely due to the fact there is no regulatory requirement for quality assurance of nicotine content. Accurate determination of nicotine content in VNP products, along with assay quality assurance is a fundamental requirement when assessing these products for harm and safety, and if they have any role in smoking reduction.

Apart from knowing the constituents of nicotine products themselves, the biological markers were used as a determinant for the intake of nicotine and smoking-related harm aimed at smoking reduction. The biological marker – NNAL – provided the analytical evidence to support the outcomes of smoking reduction. In this thesis, an efficient and easy to approach measurement assay, was developed to determine NNAL levels in urine samples. The assay was successfully applied on participants currently involved in a smoking reduction study. In addition, there were advantages associated with this newly developed liquid-liquid extraction assay, especially combined with UPLC-MS/MS. To establish the accomplished methodology, different methods were designed and compared. It was also found that new types of extraction methods, DLLME and DLLME-SFO, were not suitable for NNAL determination. There were limitations with the capacity of the extraction solvent in the DLLME method and the issue with evaporating the extraction solvent in DLLME-SFO method. In addition, simplified SPE procedures were designed, but this resulted in chromatographic signals of analyte which were embedded with the background noise signals. Therefore, the urine matrices interference was not effectively reduced with the simplified

SPE procedures. In summary, the established liquid-liquid extraction assay coupled with UPLC-MS/MS was found to be more efficient for NNAL analysis when applying NNAL as biological markers to examine the actual smoking harm exposure in clinical smoking reduction studies.

Apart from developing the assays for the measures of the constituents of nicotine products and the biological markers, a better understanding on the impact of reduction was also important. A clinical trial involving two treatments: nicotine patches and varenicline was conducted. This trial examined the effect of pre-quit treatments on smoking rate and satisfaction with smoking. The two treatments were applied two weeks prior to a target quit day (as pre-quit groups), and compared with a normal standard nicotine patch applied on the target quit day (as a control group). The study found that the satisfaction with smoking and smoking rate were significantly reduced during the pre-quit treatment period in the pre-quit treatment groups; the control group did not. Satisfaction was significantly associated with the reduction of smoking rate, although the mediation effect was relatively small. Satisfaction did not appear to be a viable method of evaluating responsiveness of treatment. Other variables, for example craving and dependence on smoking, may mediate the smoking reduction. In addition, based on the findings of the NNAL assay that we developed, there was a significant reduction in biological markers of harm after a quit attempt with using smoking treatments. For future studies, examining the relationship between cravings, dependence on smoking, and measuring the levels of biological markers may prove to be useful in evaluating responsiveness to treatment, risk of harm, and allow for tailoring of treatment.

In summary, we know that there is no known safe level of smoking: every cigarette smoked increases an individual's risk of tobacco-related disease. Tobacco-related harm

reduction strategies aim to assist people to reduce smoking. However, quantifying the risks and benefits of tobacco harm reduction, either at a socially or an individual level, is complicated, even assessing reduction itself is complicated as the exact harm associated with each cigarette is not consistent: it is a crude measure reduction by simply counting the number of CPD that an individual consumes.

To better quantify the reduction, methods of measuring biological markers of tobacco exposure and the constituents of tobacco products were developed. However, no current measure was a perfect proxy for actual exposure and there were limitations of the exist methods of measuring. Hence, this thesis aims to develop improved measures of exposure – both in terms of measuring biological markers of actual exposure, and in terms of the constituents of products themselves – and to use these measures to studying smoking behaviour and its consequences. The analytical assays of biological markers measurement were developed and successfully applied on participants in the smoking reduction study. Overall, pharmacotherapy products used as a pre-quit treatment prior to the complete cessation introduced a significant reduction in satisfaction with smoking and cigarette consumption. The biological marker – NNAL – provided the analytical evidences to support the outcomes of smoking reduction. In addition, the developed assay for determination of nicotine content in medical nicotine products may provide the implications for further study of the nicotine products and application of these products for smoking reduction.

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Appendix I: Pre-quit Treatment Study Protocol

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BMC Public Health

STUDY PROTOCOL Open Access

Examination of the mechanism of action of two pre-quit pharmacotherapies for smoking cessation

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Abstract

Background: There is substantial scope for improvement in the current arsenal of smoking cessation methods and techniques: even when front-line cessation treatments are utilized, smokers are still more likely to fail than to succeed. Studies testing the incremental benefit of using nicotine patch for 1–4 weeks prior to quitting have shown pre-quit nicotine patch use produces a robust incremental improvement over standard post-quit patch treatment. The primary objective of the current study is to test the mechanism of action of two pre-quit smoking cessation medications—varenicline and nicotine patch—in order to learn how best to optimize these pre-quit treatments.

Methods/Design: The study is a three group, randomized, open-label controlled clinical trial. Participants (n = 216 interested quitters) will be randomized to receive standard patch treatment (10 weeks of patch starting from a designated quit day), pre-quit patch treatment (two weeks of patch treatment prior to a quit day, followed by 10 weeks post-quit treatment) or varenicline (starting two weeks prior to quit day followed by 10 weeks post-quit). Participants will use study-specific modified smart-phones to monitor their smoking, withdrawal symptoms, craving, mood and social situations in near real-time over four weeks; two weeks prior to an assigned quit date and two weeks after this date. Smoking and abstinence will be assessed at regular study visits and biochemically verified.

Discussion: Understanding how nicotine patches and varenicline influence abstinence may allow for better tailoring of these treatments to individual smokers.

Trial registration: Australian New Zealand Clinical Trials Registry, ACTRN12614000329662 (Registered: 27 March 2014).

Keywords: Nicotine patch, Varenicline, Pre-quit treatment, Smoking reduction

Background

Cigarette smoking remains one of the leading causes of preventable death and disease worldwide [1, 2]. Perhaps not surprisingly, the majority of smokers indicate that they would like to quit [3]; despite this interest in quitting, however, the proportion of smokers who successfully quit each year is very low. Poor cessation rates can partly be attributed to the low uptake of efficacious smoking cessation methods [4]—a review [5] of unassisted ("cold turkey") quit attempts concluded that the vast majority end in failure; however, even when smokers adopt effective cessation methods the most likely outcome is still failure. Even with the currently accepted gold standard treatment (i.e., pharmacotherapy combined with behavioural counselling) approximately 70 % [6, 7] of quit efforts fail. Thus, not only do interested quitters eschew current smoking cessation methods more often than not, but also when they are used they afford only modest improvements in the overall likelihood of quitting. Developing efficacious treatments that are accessible to a wide range of smokers will reduce tobacco-related deaths [8].

Numerous studies have been conducted over the last two decades aimed at developing new smoking cessation methods and treatments (e.g., [9, 10]), with some notable successes [11]. An alternate approach, however, is to optimize the use of existing smoking cessation methods and agents: improvements in quit rates can come from

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innovations in the way currently available treatments are used. Nicotine patch—a form of Nicotine Replacement Therapy (NRT)—is the most widely used pharmacotherapy to aid smoking cessation [4]. Patch treatment approximately doubles a smoker's chance of maintaining longer-term abstinence (compared to placebo treatment) [7]. Nicotine patch was originally approved as an aid to abrupt cessation: smokers were instructed to stop smoking entirely and then start using the medication [7]. More recently, researchers have started to explore alternative methods of using patch, with an eye towards improving its effectiveness (e.g., [12, 13]). One alternative that has been of particular interest to researchers has been the incremental benefit of using nicotine patch for 1–4 weeks prior to quitting (so called nicotine "preloading" [14]), followed by the standard post-quit patch regime. Studies showed that nicotine patch preloading improves treatment efficacy: pre-quit nicotine patch use appears to produce a robust incremental improvement over standard patch treatment [14–16].

Why might using nicotine patch prior to quitting aid smoking cessation over and above the standard regime of starting patch on quit day? The most commonly voiced mechanism is that the non-contingently delivered nicotine from pre-quit nicotine patch treatment makes cigarettes less satisfying, thus blunting the reinforcement gained from smoking; this reduced satisfaction drives smoking reduction and subsequent cessation [14, 15]. Some support for this mechanism can be found in the nicotine patch literature. Previous studies have found that smokers rate cigarettes smoked while wearing a nicotine patch as less satisfying (e.g., [17–19]; c.f., [20]) and that the satisfaction obtained from smoking lapses predicts subsequent abstinence [20]. However, randomised studies that have examined pre-quit nicotine patch treatment's effect on smoking satisfaction during the pre-quit period have found mixed results [15].

One reason for the mixed findings could be due to methodological shortcomings of the studies conducted to date. Studies of pre-quit nicotine patch use have typically relied on a participant's retrospective recall to gather information on the effects of smoking while using patch. Such approaches are prone to systematic errors and bias [21–23]; these biases can mask legitimate trends in the data, and potentially lead to spurious conclusions.

More recently, our group conducted a small ($n = 61$) feasibility study designed to test the effect of pre-quit nicotine patch treatment on satisfaction gained from smoking. Using a single-group design [19], interested quitters monitored their smoking, affect and activities during two weeks of pre-quit nicotine patch treatment. In order to reduce the need for retrospective recall, data were collected in real-time using hand-held computers [22]. Participants were then followed-up after four weeks of post-quit treatment and abstinence was assessed. As expected, both smoking rate and satisfaction with smoking declined during the pre-quit nicotine patch period, and further analysis suggested that the degree of reduction achieved during the pre-quit period was related to the likelihood of abstinence at the four-week follow-up, although this relationship was not significant [19]. While these results are supportive of the posited mechanism of action, the absence of a control group meant that the study could not determine whether it was actually the pre-quit nicotine patch treatment that was driving the reductions in satisfaction and smoking rate observed during the pre-quit phase. The current study was designed to overcome this limitation by including a control group of participants who started nicotine patch treatment on quit day.

Nicotine patch is not the only smoking cessation pharmacotherapy to be used prior to quit: varenicline is also typically started 1–2 weeks prior to a quit attempt. Varenicline is the newest pharmacological smoking cessation agent and has been found to be more effective than other mono-therapies [6]. Interestingly, studies (e.g., [24]) have suggested that, like pre-quit nicotine patch treatment, the efficacy of varenicline may also, at least in part, be mediated via reductions in the satisfaction gained from smoking. Understanding how these two pre-quit agents influence abstinence may allow for better tailoring of treatment (e.g., if the proposed mechanism of action is confirmed, it may be beneficial to tailor the length of the pre-quit treatment phase until sufficient reduction has occurred, or to use reduction as an early indicator of treatment effectiveness [25]). Furthermore, understanding the effects of pre-quit treatment may provide targets for new medications.

The study will use a three-group, randomised, open-label study design. Participants will be randomized to one of two pre-quit treatment conditions, or a control group. Participants in the active pre-quit conditions will received two weeks of active medication treatment (nicotine patch or varenicline) prior to an assigned quit day, followed by up to 10 weeks of post-quit treatment; participants randomized to the control group will receive up to 10 weeks of nicotine patch treatment starting from their assigned quit day (so no treatment during the pre-quit phase). Using this design we plan to test four hypotheses: 1) Participants in the pre-quit treatment conditions will experience a significant reduction in the satisfaction gained from smoking over the two-week pre-quit period (compared to participants in the control group); 2) Participants in the pre-quit treatment conditions will experience a significant reduction in craving over the two-week pre-quit period (compared to participants in the control group); 3) Participants in the pre-quit treatment

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conditions will significantly reduce smoking rate over the two-week pre-quit period (compared to participants in the control group); and 4) Smoking reduction during the pre-quit phase will be positively associated with abstinence at four-week follow-up. A secondary objective of the study will be to look for moderators of treatment outcome (e.g., nicotine metabolism [26]) and to explore the effect of pre-quit treatment on lapses that occur post-quit.

Methods

Overview

Interested quitters will be monitored for two weeks before, and four weeks after, a quit attempt. Participants will be randomized to a Varenicline Group, a Pre-Quit Patch Group, or a Standard Patch Group. Participants randomized to the Standard Patch Group will receive nicotine patch treatment starting from their assigned quit day. Participants in the Pre-Quit Patch and Varenicline groups will receive two weeks of active medication treatment prior to an assigned quit day, followed by up to 10 weeks of post-quit medication. All participants will use a customized smart-phone [22] to monitor their smoking, affect, and activities in real-time for four weeks (two weeks prior to an assigned quit date and two weeks after this date). Abstinence will be assessed at regular study visits.

Design

The study will use a three group, randomized, open-label controlled clinical trial study design.

Participant recruitment

A community sample of interested quitters will be recruited from the greater Hobart (Tasmania, Australia) region using advertisements in local newspapers, flyers, and through targeted advertisements on social media websites such as Facebook [27].

Inclusion/Exclusion criteria

To be eligible for enrolment, interested individuals will have to report: a) being ≥18 years old; b) smoking ≥10 cigarettes per day for the past three years; c) having a high motivation to quit smoking; d) be willing to use either patches or varenicline as part of a quit attempt; e) being able to read and write English; and f) be willing to consent to and complete the research tasks. Potential participants will be excluded if they: a) are currently enrolled in a smoking cessation trial, or have been within the past three months; or b) are unsuitable for treatment with either patches or varenicline (per current Australian prescribing guidelines [28]). Specifically, interested smokers will be excluded if they: Are a diabetic; have a mental illness or a history of repeated fits or convulsions (epilepsy); have kidney or liver problems; have a skin condition or disease such as allergic eczema or dermatitis, or allergies to any other medicines such as an itchy skin rash or swelling of the lips, face and throat; have uncontrolled, overactive thyroid gland; have a history of heart problems such as heart attack, chest pain or stroke, or untreated high blood pressure; or, are currently pregnant or breastfeeding. Initial screening will be conducted by phone and will be repeated in person by a trained study staff at the enrolment session. Screening will be overseen by a qualified general practitioner.

Interventions

All participants will receive a standard paper-based self-help quitting booklet, provided prior to an assigned quit day. Participants randomized to either of the two pre-quit groups will receive medication for two weeks of active treatment (nicotine patches or varenicline) prior to their assigned quit day, followed by up to 10 weeks of post-quit treatment. Participants in the Standard Patch Group will start active patch treatment on the morning of their assigned quit day; they will not receive any medication during the pre-quit period. Medication (patches or varenicline) will be distributed at study visits (see below), with participants being provided enough medication to last until the next scheduled visit.

As all participants must report smoking ≥10 cigarettes per day to be eligible for enrolment, patch dose (for Pre-Quit Patch and Standard Patch participants) will be determined by baseline body weight per Australian guidelines [28]: participants who report a weight of ≥45 kg at enrolment will start on 21 mg/24-hr patches while participants who report a weight of <45 kg at enrolment will start on 14 mg/24-hr patches. Post-quit, participants will remain on the same dose for 10 weeks; dose will not be titrated downwards over the course of the study (again, in line with Australian guidelines). Participants in the Varenicline Group will receive 1 mg twice per day following a one-week titration period (0.5 mg once per day for three days, followed by 0.5 mg twice per day for four days). During each study visit participants will be asked about medication use since the last study visit (including frequency of use and adverse events) and any unused medication will be collected.

Trial procedures

All participants will be asked to attend up to five study visits and to monitor their smoking using a modified smart-phone for a total of four weeks. Primary data collection will take place via the customized smart-phone (described below), with additional information obtained via questionnaires at study visits. All measures have been used and validated in the context of previous smoking studies.

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At the beginning of the enrollment visit (Visit 1) eligible and interested smokers will be given a detailed overview of the study requirements and asked to provide written informed consent. Participants will then be randomized to their treatment group and assigned a quit date that will fall 17 days after initial enrolment. Participants will be instructed to quit smoking completely on their assigned quit day. During the initial visit participants will also provide two expired air carbon monoxide (CO) samples (see below), and a urine sample.

Baseline questionnaire assessment

During the initial visit, participants will be asked to complete a detailed smoking history and demographic characteristics questionnaire. Participants will be asked about basic demographic characteristics (e.g., age, gender, education, income, occupation, ethnicity, marital status etc.), current smoking and smoking history (e.g., age at initiation, cigarettes per day [CPD], years smoked, interest in quitting, past quit efforts etc.), nicotine dependence (as assessed by Fagerström Test for Nicotine Dependence [29] and the Nicotine Dependence Syndrome Scale [30]), alcohol use [31], feelings of depression [32], and satisfaction gained from cigarettes (using the modified Cigarette Evaluation Questionnaire [mCEQ]; [33]). As well as helping to characterize our sample, responses to these items will be used in our exploratory analyses of moderators of treatment outcome.

Ecological Momentary Assessment (EMA) data collection procedures

To collect near real-time data from participants as they undertake their quit effort, participants will monitor their smoking along with other study variables using modified smart-phones. At Visit 1 all participants will be provided with a smart-phone that has been stripped of its native functionality. These devices will be loaded with custom EMA data collection software designed by the study team. Participants will be asked to carry this device with them at all times for the following four weeks (until Visit 4). Participants will be asked to indicate, by pressing a button on the phone, each time they smoke a cigarette. The device will log the time and date of these events and store this data for later download and analysis.

During the pre-quit monitoring phase (i.e., Visit 1–3), the phone will be used to administer three types of assessments. Firstly, following 4–5 randomly selected cigarette reports per day, participants will be asked to complete an assessment of their current state (mood, withdrawal severity, craving etc.) as well as contextual and situational details (where the participants is, who they are with, what they are doing etc.). In addition to these "smoking assessments" the device is programmed to "beep" participants at a rate of approximately 4–5 times per day for randomly-selected non-smoking assessments ("random assessments"). During these assessments, participants will complete a series of items that parallels those administered during smoking assessments. Finally, participants will also complete end-of-day assessments to gather global reports on daily mood, craving and quitting self-efficacy. The two weeks of post-quit EMA monitoring will closely mirror that used in baseline monitoring, however post-quit assessments will include additional items pertaining to the lapse trigger(s) and the use (if any) of coping mechanisms [21]. Items in the proposed EMA assessments have been used and validated in previous EMA studies, and are reported in detail in resulting publications [21, 34]. Participants will be trained in EMA procedures and on assessment content before field monitoring commences formally (during Visit 1). Following procedures established in previous successful EMA studies [21], the second study visit (Visit 2) will be scheduled 2–4 days after participants start EMA field monitoring, at which time their data will be downloaded and checked, and they will receive further EMA training (if necessary). At the end of EMA monitoring (Visit 4) the study phones will be retrieved and re-used with subsequent participants.

Study visits 2–5

Participants will complete a further 4 study visits as part of the study. During each of these visits, CO and urine samples will be obtained for all participants, and satisfaction with smoking will be assessed using the mCEQ. During Visit 2 (14 days prior to quit day), participants in the Standard Patch group will be provided with one patch, which they will be instructed to apply on the morning of their assigned quit day. EMA data will be downloaded from all participants and checked for compliance, with extra training provided if necessary. Visit 3 will occur on a participants target quit day (17 days after enrollment). During this visit, in addition to the general tasks described above EMA data will be downloaded and checked for protocol compliance with additional training provided if necessary.

Study Visit 4 will occur 14 days after quit day and will mark the end of the EMA monitoring period and all phones will be handed back. The final study visit (Visit 5) will occur 28 days after participants target quit day. Participants who self-report 7-day point prevalent abstinence at this visit, verified by expired air CO (see below), will be provided a further six weeks of study medication. In addition to the general study visit tasks, during their final study visit participants will then complete an exit interview during which they will be asked about the study interventions and their experiences in the study. They will also be fully debriefed and offered the opportunity to ask any final questions about the study.

Compensation and retention

In addition to receiving treatment, participants who complete the entire study will be paid $50. Participants will be paid for each visit that they complete, with the per visit payment escalating over the course of study visits ($10 at Visit 1, $20 at Visit 3, $20 at Visit 5). Once enrolled, participants will be sent a reminder e-mail and/or telephoned 24 h before a scheduled appointment. At the end of each session, the next session will be scheduled.

Verification of smoking status

At each study visit participants will be asked about their current smoking status and any smoking that has occurred since the previous study visit (via a calendar-assisted 14-day timeline follow-back questionnaire). To verify self-report, expired air CO samples will be obtained at each study visit using a MICRO+ Smokerlyzer® (Bedfort Scientific, UK). Two samples will be recorded at each visit and if the average CO concentration of the two samples is <8 ppm, this will be taken as evidence of abstinence.

Analytic plan

The proposed sample size ($n = 216$; 72 participants per group) was determined by the requirements of the primary research questions—namely, detecting reductions in satisfaction (H1) and craving (H2) scores, and smoking rate (H3), between the Standard Patch and the two pre-quit treatment groups at the end of the pre-quit treatment period (Visit 3). In a previous study [19] we observed moderate-to-large effects of pre-quit nicotine patch treatment on self-reported satisfaction with smoking, craving, and smoking rate during the pre-quit treatment period. Using these reductions as a proxy for between-group differences (assuming that these measures will not decrease during the pre-quit period for participants in the Standard Patch group), a conservative estimate of within-subject correlation of measures (0.5), our proposed sample will afford >90 % power to detect a difference in these measures. Our proposed sample size will also afford >80 % power to detect an effect of smoking reduction during the pre-quit phase on treatment outcomes (assessed at Visit 5; H4). Based on an earlier study [19] we expect to see ~6 % dropout before Visit 3 and as such we aim to recruit and enroll 229 participants in order to achieve our evaluable sample.

Our primary research questions involve reductions in satisfaction with smoking (H1), cigarette craving (H2), and smoking rate (H3) during the pre-quit treatment phase. These questions will be tested separately for each of the pre-quit groups using data gathered from the EMA field monitoring using mixed models growth curve analyses. The effect of smoking reduction on abstinence (H4) will be assessed in two ways. Firstly, reduction during the pre-quit phase will be used in a logistic regression model to predict biochemically verified 7-day point prevalent abstinence at Visit 5 (4 week abstinence). Treatment group will be included as a covariate. This outcome analysis will be conducted as intent-to-treat, with all participants who are randomized (Visit 2) being included in the analyses. Study dropouts will be counted as treatment failures. Next, as traditional intent-to-treat point prevalent abstinence analyses can obscure important aspects of the process of quitting smoking [13, 35], we will also use the real-time EMA data to examine time to first lapse using survival analyses.

In an exploratory analysis, we will compare the context and consequences of smoking lapses across the three treatment groups using data gathered during real-time assessments of smoking lapses [34]. Finally, we will examine whether individual differences in nicotine metabolism moderate treatment outcome. Nicotine metabolism will be expressed as the ratio of 3′-hydroxycotinine to cotinine in urine, as well as directly measuring their respective glucuronides (cotinine-N-glucuronide and 3′-hydroxycotinine-O-glucuronide). Instrumental analysis of urines will be undertaken using an Ultra Performance Liquid Chromatography-tandem mass spectrometry [36]. The main outcome measure will be total levels (free + conjugated) of cotinine and 3′-hydroxycotinine.

Discussion

This study is designed to test the mechanism(s) though which pre-quit treatments—specifically nicotine patches and varenicline—aid cessation. Understanding how these two pre-quit treatments influence abstinence may allow for better tailoring of these efficacious treatments. Additionally, understanding the effects of pre-quit treatment with nicotine patch and varenicline may provide targets for new pharmacotherapies.

Ethics and research governance

The project was reviewed and approved by the Tasmanian Health and Medical Research Ethics Committee (H0013619) prior to starting the recruitment process. Written informed consent will be obtained from all participants.

Competing interests

SGF has worked as a consultant for pharmaceutical companies on matters relating to smoking cessation. This project is supported by a researcher-initiated project grant from Pfizer (through the GRAND initiative) awarded to SGF.

Authors' contributions

SGF conceived the study. SGF, JAEW and NS were involved in the initial discussions that led to the grant application, writing of the study protocol and participated in the study design. SGF devised the statistical analysis plan. WL designed the urine analyses. All authors contributed to the draft of the manuscript. All authors read and approved the final version of the manuscript.

Appendix I

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